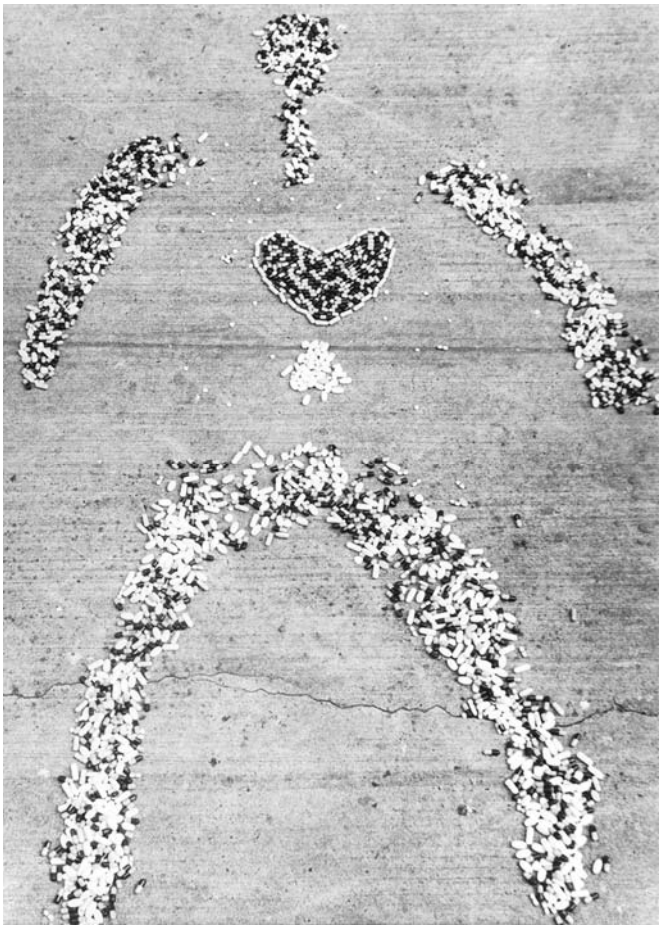
Evaluation of HIV-1 viral load and genotypic resistance assays for resource-limited settings



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Cover illustration

‘Three Month Supply’ by Max Greenberg, an HIV-positive artist

'This is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.'

Winston Churchill, London, 10 November 1942

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Table of contents

Abbreviations

3TC	lamivudine
ABC	abacavir
Ag	antigen
AIDS	acquired immune deficiency syndrome
ANRS	agence national de la recherche sur le SIDA
APV	fosamprenavir
ART	antiretroviral treatment
ARV	antiretroviral
ATV	atazanavir
AZT	zidovudine
c/ml	copies per ml
CCC	comprehensive care centre
CPGH	coast province general hospital
CRFs	circulating recombinant forms
d4T	stavudine
DBS	dry blood spots
ddC	zalcitabine
ddI	didanosine
DHHS	department of health and human services
DLV	delavirdine
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DRV	darunavir
EFV	efavirenz
ELISA	enzyme linked immunosorbent assay
ENF	enfuvirtide
env	envelope
FDA	food and drug administration
FTC	emtricitabine
gag	group specific antigen
gp	glycoprotein
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
IC_{50}	50% inhibitory concentration
ICRH	international centre for reproductive health
IDV	indinavir
IN	integrase
kb	kilobases

KS	Kaposi's Sarcoma
LPV	lopinavir
MA	matrix proteins
MVC	maraviroc
NC	nucleocapsid
Nef	negative factor
NFV	nelfinavir
NGO	non governmental organization
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside/nucleotide reverse transcriptase inhibitors
NVP	nevirapine
ORFs	open reading frames
p	protein
PBMCs	peripheral blood mononuclear cells
PCP	*Pneumocystis Carinii* Pneumonia
PCR	polymerase chain reaction
PIs	protease inhibitors
pol	polymerase
PR	protease
RAL	raltegravir
Rev	regulator of viral protein synthesis
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
RTV	ritonavir
SU	surface unit
SQV	saquinavir
STIs	sexually transmitted infections
Tat	transcriptional transactivator
TDF	tenofovir
TDR	transmitted drug resistance
TLC	total lymphocyte count
TM	transmembrane unit
TPV	tipranavir
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
WHO	World Health Organization

Summary

HIV/AIDS remains the world's leading infectious cause of death, despite global efforts for prevention and better access to treatment. At the end of 2007, about 33 million people were living with HIV/AIDS, with nearly 2.5 million newly infected yearly and Sub-Saharan Africa being most affected. Although the use of ART has significantly reduced HIV-related mortality in the industrialised world, only 28% of people in need of ART worldwide, actually have access to it. Global initiatives aiming at universal access to ART by 2010 are still ongoing. The expansion of global access to ART should remain a priority, yet tools to assess the response to treatment are equally important. Assays to quantify the viral burden in plasma and to identify drug resistant mutations have become the standard of care to monitor patients on treatment in the industrialised world. However, widespread use of these assays in resource-limited settings is still hampered by their high cost and the lack of well-equipped laboratories. Therefore, there is an urgent need to develop and evaluate simple and affordable alternatives for the current viral load and resistance assays, which can be implemented in resource-limited settings.

Regular viral load measurements are important parameters to detect early treatment failure, thereby avoiding the accumulation of drug resistance mutations. They can also diagnose perinatal infections and prevent unnecessary treatment switches, by monitoring adherence to ART. Moreover, viral load assays could be useful as a sentinel surveillance tool in ART program quality assessments. WHO recognises the need for viral load assays and advocates the use of simple, inexpensive and accurate tools for measuring HIV-1 viral load. Three types of alternative viral load assays have been proposed: assays to quantify p24 Ag, assays to measure reverse transcriptase activity and in-house real-time PCR assays. The latter two showed the most promising results, however little information about performance of these assays in a resource-limited setting is available. Therefore we decided to assess the usefulness of the ExavirLoad (Cavidi) and Generic HIV viral load (Biocentric) in a real-life situation in Mombasa, Kenya. We found a good correlation for both assays when compared with the Amplicor HIV-1 Monitor v1.5 assay (Roche), but the specificity was less optimal, especially for the ExavirLoad. Both these assays could be valuable tools for viral load measurements, yet they have their drawbacks and the

choice for the right viral load assay should be made at each site individually, taking the specific advantages and disadvantages into account.

The lack of affordable viral load measurements as a parameter to assess early treatment failure, will increase the risk of accumulation of resistance mutations leading to a high probability of cross-resistance. This will not only pose a problem for the individual patient, but it will also increase the risk of transmission of highly resistant viruses in the population, jeopardizing future treatment options. There is an urgent need for affordable genotypic resistance assays and therefore an in-house genotyping assay was evaluated. Due to the high sensitivity of this assay, it allows the combined detection of plasma HIV-1 and possible drug resistant mutations, reducing the need for standard viral load and genotyping assays.
Widespread use of viral load testing and genotyping in resource-limited settings, is not only hampered by the high cost of these assays but also by the lack of proper infrastructure. One of the challenges is to guarantee the cold chain, needed for the storage and transportation of blood samples. In this respect our in-house genotyping assay was adapted for the use of cellular DNA from whole blood and dried blood spots (DBS). Our study showed the feasibility of extracting and subsequently sequencing HIV-1 DNA from whole blood and DBS. Although DNA sequencing can be useful in epidemiological studies, such as analysing subtype distribution and the overall spread of drug resistance mutations, our study confirmed that RNA sequencing remains superior over DNA sequencing in the individual follow-up of patients, especially at early treatment failure.

The scale-up of ART in low and middle-income countries has been proven successful, despite the lack of adequate laboratory assays to monitor patients on treatment. Similarly, we found a treatment success rate of 86.2% for those treated for at least 6 months at the Comprehensive Care Centre in Mombasa. However, the rate of accumulation of resistance mutations among the patients failing treatment is of great concern. In our study, 87.5% of the patients with a detectable viral load, developed resistance against reverse transcriptase inhibitors. Even more worrying is the observation that 62.5% of the patients failing treatment, developed multi-class resistance. This will significantly impact future treatment options, especially because only few drugs for second-line treatment are available in resource-limited settings.

Regular viral load measurements can avoid the accumulation of drug resistance and, combined with adherence counselling, they can increase the chance to remain on a first-line regimen for a longer period.

Global efforts to provide universal access to ART should continue, despite the limited possibility to adequately monitor patients on treatment in low and middle-income settings. Yet, the need for affordable and accurate viral load and resistance assays should be emphasized. Researchers and commercial companies should continue their search for low-cost laboratory assays to monitor HIV-1 patients on treatment and more generic ARV drugs should be made available in resource-limited settings in order to improve the worldwide HIV care.

Samenvatting

Ondanks wereldwijde inspanningen voor de preventie van HIV infectie en het verbeteren van de beschikbaarheid van antiretrovirale therapie (ART), blijft HIV/AIDS de belangrijkste infectieuze doodsoorzaak ter wereld. Eind 2007 werden al meer dan 33 miljoen mensen getroffen door HIV/AIDS, waaronder 2.5 miljoen nieuwe infecties. Sub-Saharisch Afrika blijft het meest getroffen gebied. Alhoewel het gebruik van ART de HIV gerelateerde mortaliteit enorm heeft doen dalen in de geïndustrialiseerde wereld, hebben wereldwijd slechts 28% van de mensen die therapie nodig hebben, ook toegang tot de medicatie. Ondertussen werden er wereldwijde initiatieven opgezet om ART universeel toegankelijk te maken tegen 2010. De uitbreiding van wereldwijde beschikbaarheid van ART moet een prioriteit blijven, maar de testen die nodig zijn om de respons op de behandeling te meten zijn minstens even belangrijk. Het meten van de virale lading en het opsporen van resistentie zijn standaard testen in ontwikkelde landen. Het gebruik van deze testen in ontwikkelingslanden blijft echter beperkt door de hoge kost en het gebrek aan goed uitgeruste laboratoria. Daarom is er een hoge nood om eenvoudige en betaalbare alternatieven voor de huidige virale lading en resistentietesten te ontwikkelen en te valideren, die dan geïmplementeerd kunnen worden in ontwikkelingslanden.

Het regelmatig meten van de virale lading is een belangrijke parameter om vroeg therapiefalen op te sporen. Hierdoor kan de accumulatie van mutaties die tot resistentie leiden, vermeden worden. Het meten van de virale lading is ook bruikbaar voor het diagnosticeren van perinatale infecties en als parameter voor therapietrouw kunnen ze onnodige therapie wijzigingen voorkomen. Bovendien kunnen deze testen gebruikt worden om de efficiëntie van ART programma's te evalueren. De WHO erkent de nood voor deze testen en pleit voor het gebruik van eenvoudige, goedkope en accurate testen voor het meten van de HIV virale lading. Er werden reeds drie types van alternatieve testen voorgesteld: testen om p24 Ag te bepalen, testen die de activiteit van het reverse transcriptase meten en 'in-house' real-time PCR testen. De laatste twee geven de meest belovende resultaten, maar over de bruikbaarheid ervan in ontwikkelingslanden is nog niet veel geweten. Daarom besloten we om het nut van de ExavirLoad (Cavidi) en de Generische HIV virale lading (Biocentric) te evalueren in Mombasa, Kenia. Onze studie toonde aan dat beide testen goed correleerden met de Amplicor HIV-1 Monitor v1.5 (Roche), maar dat de specificiteit

inferieur was aan deze laatste, vooral in het geval van de ExavirLoad. Beide testen kunnen echter waardevol zijn voor het bepalen van virale ladingen, maar hebben elk hun nadelen en daarom moet de keuze voor de juiste test in iedere setting apart gemaakt worden, rekening houdend met de specifieke voor- en nadelen.

De accumulatie van verschillende met resistentie geassocieerde mutaties, met vaak kruisresistentie tegen verschillende producten tot gevolg, is niet enkel een probleem voor de individuele patiënt, maar verhoogt ook het risico op overdracht van resistent virus en dus de verspreiding van resistentie in de populatie. Er is bijgevolg nood aan betaalbare genotypische resistentietesten. De hoge gevoeligheid van onze 'in-house' genotypische test laat de gecombineerde detectie van plasma HIV-1 en opsporing van resistentiemutaties toe.
Het wijdverspreide gebruik van testen voor virale lading en genotypering in ontwikkelingslanden is niet enkel gelimiteerd door de hoge kostprijs, maar ook door het gebrek aan infrastructuur. Eén van de uitdagingen is het garanderen van een koude keten die nodig is voor het bewaren en transporteren van bloedstalen. In dit opzicht hebben we de 'in-house' test voor resistentiebepaling aangepast voor gebruik van cellulair DNA uit vol bloed en droge bloed spots (DBS). We konden aantonen dat het extraheren en vervolgens sequeneren van HIV DNA uit vol bloed en DBS mogelijk is. De resultaten bekomen via DNA sequenering zijn bruikbaar voor epidemiologische studies, zoals het analyseren van de HIV subtype-verspreiding en van de algemene verspreiding van resistentiemutaties. We konden echter ook aantonen dat bij het opvolgen van individuele patiënten, het sequeneren van RNA veel meer informatie geeft dan het sequeneren van DNA, vooral bij vroeg therapiefalen.

Ondanks het gebrek aan goede laboratoriumtesten om behandelde patiënten op te volgen, zijn de inspanningen om ART meer toegankelijk te maken voor HIV-patiënten in ontwikkelingslanden niet zonder resultaat. In Mombasa vonden we dat 86.2% van de patiënten, die voor minstens 6 maanden behandeld werden, een niet detecteerbare virale lading bereikten. Nochtans is het aantal falende patiënten met resistentie zorgwekkend. In onze studie ontwikkelden 87.5% van de patiënten met een detecteerbare virale lading resistentie tegen reverse transcriptase inhibitoren. Het feit dat 62.5% van de falende patiënten resistentie ontwikkelden tegen meerdere

klassen van geneesmiddelen is nog meer verontrustend. Dit zal een belangrijke invloed hebben op toekomstige therapie mogelijkheden, vooral omdat er in ontwikkelingslanden slechts een beperkt aantal geneesmiddelen beschikbaar zijn voor tweedelijns therapie. Regelmatige metingen van de virale lading kunnen de accumulatie van resistentie beperken en kunnen, in combinatie met verbeterde therapietrouw, de kans om de patiënten langer op een eerstelijns therapie te houden, verhogen.

Ondanks de beperkte mogelijkheden om patiënten op therapie op te volgen in ontwikkelingslanden, moeten we de universele beschikbaarheid van ART blijven promoten. Anderzijds blijft de nood aan betaalbare virale lading en resistentietesten enorm hoog. Onderzoekers en commerciële firma's moeten blijven volharden in hun zoektocht naar goedkope laboratoriumtesten om HIV geïnfecteerde patiënten op therapie op te volgen. Bovendien moeten meer generische geneesmiddelen beschikbaar gemaakt worden in ontwikkelingslanden om de zorg voor HIV geïnfecteerden wereldwijd verder te verbeteren.

Résumé

Malgré des efforts importants de prévention et pour améliorer l'accès au traitement, le VIH et le SIDA demeurent la principale cause de mortalité par maladie infectieuse. Fin 2007, environ 33 millions de personnes vivaient avec le VIH ou le SIDA; chaque année, environ 2.5 millions de personnes sont nouvellement infectées, l'Afrique Subsaharienne étant la région la plus affectée. Dans les pays industrialisés, l'utilisation du traitement antirétroviral (TAR) a considérablement réduit la mortalité liée au VIH. Mais au niveau mondial, seuls 28% des personnes nécessitant le TAR ont dans les faits accès à celui-ci. Différentes initiatives visant à un accès universel au TAR d'ici à 2010 sont en cours au niveau mondial. L'élargissement de l'accès au TAR au niveau mondial doit rester une priorité. Cependant, il est tout aussi important de disposer d'outils permettant d'évaluer la réponse au traitement. Dans les pays industrialisés, différents tests permettant de mesurer la charge virale et d'identifier les mutations entraînant des résistances aux médicaments font maintenant partie de la prise en charge standard. Dans les situations de ressources limitées, l'utilisation à grande échelle de ce type de tests est cependant limitée par leur prix élevé et parce qu'ils ne peuvent être utilisés que dans des laboratoires bien équipés. Il y a donc un besoin urgent de développer et d'évaluer des méthodes alternatives de mesure de la charge virale et de détection des résistances qui soient simples et bon marché et puissent être mises en œuvre dans les situations de ressources limitées.

La mesure régulière de la charge virale est un paramètre important pour détecter de façon précoce un échec thérapeutique, permettant de ce fait d'éviter la multiplication des mutations à l'origine de résistance, et, en cas de montée de la charge virale, d'insister à nouveau sur le conseil en matière d'observance du traitement, prévenant ainsi des changements de traitement. Cette mesure permet aussi de poser le diagnostic d'infection périnatale. Les tests de charge virale peuvent également être des outils utiles pour la surveillance sentinelle dans les programmes de TAR. L'OMS reconnaît qu'il y a un besoin de tests de charge virale, et encourage pour la mesure de la charge virale l'utilisation d'outils simples, peu coûteux et précis. Trois différentes alternatives de tests ont été proposées: les tests de quantification de l'Ag p24, les tests de mesure de l'activité de la transcriptase inverse, et les tests de PCR en temps réel de fabrication interne. Ces deux dernières alternatives de test ont donné des résultats très encourageants. On ne dispose cependant que de peu

d’information sur les performances de ces tests en situation de ressources limitées. Nous avons donc décidé d’évaluer l’utilité d’ExavirLoad (Cavidi) et de Generic HIV viral load (Biocentric) en situation de vie réelle à Mombasa, Kenya. Nous avons retrouvé une bonne corrélation de ces deux tests en les comparant au test Amplicor HIV-1 Monitor v1.5 (Roche). La spécificité était cependant moins bonne, notamment pour le test ExavirLoad. Ces deux tests pourraient être des outils précieux pour la mesure de la charge virale. Ils ont pourtant certains inconvénients. Le choix du test pour mesurer la charge virale devrait donc être fait individuellement pour chaque site, en prenant en compte les avantages ou les inconvénients de chacun de ces tests.

Ne pas disposer de la charge virale (par manque de méthodes abordables pour la mesurer) comme paramètre pour évaluer de façon précoce l’échec thérapeutique augmentera le risque d’accumuler des mutations entraînant des résistances, entraînant de ce fait un haut risque de résistances croisées. Ceci posera non seulement un problème individuel au niveau du patient, mais augmentera aussi le risque de transmission de virus hautement résistants dans la population, ce qui peut compromettre les futures options de traitement. Il est donc urgent de pouvoir disposer de tests de résistance génotypique abordables; un test génotypique de fabrication interne a donc été évalué. Ce test présente une sensibilité élevée, ce qui permet à la fois la détection du VIH-1 dans le plasma et celle d’éventuelles mutations entraînant des résistances aux médicaments; on aurait alors moins besoin de tests standard de mesure de la charge virale et de tests génotypiques. Leur coût élevé, mais aussi le manque d’infrastructures suffisantes, limite l’utilisation à grande échelle des tests de charge virale et du génotypage dans les situations de ressources limitées. L’un des défis posés est la nécessité de garantir la chaîne du froid nécessaire lors du stockage et du transport des échantillons de sang. Notre test de fabrication interne de génotypage a été adapté pour l’utilisation d’ADN cellulaire provenant de sang complet ou de gouttes de sang séchées. Notre étude a montré qu’il est possible puis de séquencer l’ADN du VIH-1 provenant de sang complet ou de gouttes de sang séchées. Le séquençage d’ADN peut être utile pour des études épidémiologiques, comme l’analyse de la distribution des sous-types et de la distribution des mutations entraînant des résistances aux médicaments. Notre étude confirme que le séquençage de l’ARN demeure supérieur à celui de l’ADN pour le suivi individuel des patients, notamment en cas d’échec thérapeutique.

Même s'il l'on manque de tests biologiques adéquates pour faire le suivi des patients sous traitement, il est maintenant démontré qu'il est possible d'élargir l'accès au TAR dans les pays à revenus faibles ou modérés. Dans les Centre de traitement global (Comprehensive Care Centre) à Mombasa, nous avons ainsi retrouvé un taux de succès thérapeutique de 86.2% chez les patients traités au cours des 6 derniers mois. Parmi les patients en échec thérapeutique, le taux d'accumulation de résistance entraînant des mutations demeure un problème. Dans notre étude, 87.5% des patients présentant une charge virale détectable présentaient une résistance aux inhibiteurs de la transcriptase inverse. Nous avons également observé que 62.5% des patients en échec thérapeutique présentaient une résistance de type multi-classe, ce qui est encore plus préoccupant. Ceci aura un impact important sur les futures options de traitement, notamment du fait que l'on ne dispose que de quelques médicaments de deuxième intention dans les situations de ressources limitées. Une mesure régulière de la charge virale peut permettre d'éviter l'accumulation de résistances. Combinée à un conseil en matière d'observance du traitement, elle peut aider à ce que les patients aient plus de chances de demeurer sous un traitement de première intention.

Il est nécessaire de poursuivre les efforts au niveau mondial pour permettre un accès universel au TAR, malgré les possibilités limitées d'offrir un suivi adéquat aux patients le recevant dans les situations de revenu modérés ou limités. Il faut cependant insister sur le fait que l'on a un grand besoin de tests de mesure de la charge virale et de détection de résistances qui soient abordables et précis. Les chercheurs et les firmes doivent continuer leurs recherches pour des tests bon marché permettant le suivi des patients infectés avec le VIH-1 sous traitement. Afin d'améliorer la prise en charge au niveau mondial, des médicaments antirétroviraux génériques doivent être mis à la disposition dans les situations à ressources limitées.

Mukhtasari (Summary Kiswahili)

Ukimwi bado ndiyo maambukizi yanayoongoza katika kusababisha vifo licha ya jitihada za ulimwengu mzima za kukinga maambukizo hayo na kupatikana kwa huduma nzuri za matibabu. Kufikia mwisho wa mwaka wa 2007, takribani watu milioni 33 walikuwa wanaishi na virusi na kuugua ukimwi, huku wengine milioni 2.5 wakipata maambukizo mapya kila mwaka na eneo la kusini mwa Sahara ya Africa likiathirika zaidi. Ijapokuwa matumizi ya madawa yanayopunguza makali ya virusi (ART) yamechangia sana kwa kupunguza idadi ya vifo vinavyotokana na ukimwi katika nchi zilizo na ustawi wa viwanda, ni asilimia 28 tu ya watu wanaohitaji ART ulimwenguni wanazipata. Mikakati ya kimataifa inayolenga kupatikaniwa ART kwa wote wanaozihitaji kufikia mwaka wa 2010 bado inaendelea. Upanuzi wa kupatikana kwa ART kwa wote ulimwenguni ni lazima ubaki kama jambo linalotiliwa maanani sana, huku vifaa vinavyotumiwa kukadiria ufanisi wa matibabu vikiwa na umuhimu sawa. Vipimo vya kupimia wingi wa virusi katika damu na kutambua virusi sugu kwa matibabu vimekuwa ndiyo kiwango cha kadri cha utunzaji katika ulimwengu uliyo na ustawi wa viwanda. Hata hivyo matumizi mengi ya hivi vipimo katika sehemu zenye uhaba wa bidhaa bado yanakabiliwa na bei za juu za vipimo na mahitaji ya mahabara zilizo na vifaa kikamilifu. Kwa hivyo kuna dharura ya kuboresha na kuzijaribu njia rahisi na ambazo watu wanaweza kumudu za kupimia wingi wa virusi na kutambua virusi sugu, ambazo zinaweza kutumika katika sehemu zenye upungufu wa vifaa.

Upimaji wa mara kwa mara wa wingi wa virusi ni hatua muhimu ili kutambua mapema kushindwa kwa matibabu, ili kuepukana na urundikaji wa virusi sugu kwa matibabu. Wanaweza pia kupima maambukizo wakati wa kuzaa ili kuepusha mabadiliko ya matibabu yasiyostahili, kwa kufuatilizia uendelezaji wa matumizi ya ART. Zaidi ya hayo, vipimo vya wingi wa virusi vinaweza kuwa vyenye manufaa kama vifaa vya kuthamini ubora wa mpango wa ART. Shirika la afya duniani linatambua haja ya vipimo vya wingi wa virusi na linahimiza matumizi ya vifaa vya kawaida, visivyo ghali na vinavyoaminika kwa kupima wingi wa virusi vya ukimwi-1. Aina tatu za vipimo vingine vinavyoweza kutumika vimependekezwa: Vipimo vinavyoweza kujumuisha p24 Ag, vipimo vya kupima transcriptase activity na in-house real-time PCR. Hivi viwili vya mwisho vilionyesha majibu yenye matumaini zaidi, hata hivyo maelezo machache tu juu ya jinsi hivi vipimo vinavyofanya kazi

katika sehemu zilizo na upungufu wa bidhaa yanapatikana. Kwa hivyo tumeamua kuangalia umuhimu wa ExavirLoad (Cavidi) na Generic HIV viral load (Biocentric) katika hali halisi ya maisha hapa Mombasa, Kenya. Tulipata uhusiano mzuri kwa vipimo vyote viwili vikilinganishwa na kipimo cha Amplicator HIV-1 Monitor v1.5 (Roche), lakini umaalumu ulikuwa mchache, haswa kwa Exavirload. Hivi vipimo vyote viwili vinaweza kuwa vifaa muhimu vya kupimia wingi wa virusi, hata hivyo vina ubaya wake na uchaguzi wa kipimo sawa lazima ufanyike katika kila kituo, ukizingatia uzuri na ubaya wa kila kifaa.

Ukosefu wa vipimo vya kupimia wingi wa virusi vya bei nafuu kama hatua ya kukadiria kushindwa kwa matibabu kwa mapema, kutaeneza athari za kuongezeka kwa virusi sugu kunaosababisha uwezekano wa juu wa cross-resistance. Hii haitaleta tu matatizo kwa mgonjwa huyu binafsi, lakini pia itaongeza athari za kusambaza virusi ambavyo ni sugu sana kwa jamii, ambapo itakuwa inakatiza njia za baadaye za matibabu. Kuna mahitaji ya haraka ya kuwa na vipimo vya bei nafuu vya kupimia usugu na kwa hivyo kipimo cha in-house genotyping kilijaribiwa. Kwa sababu ya ubora wa kifaa hiki, kinachoruhusu muungano wa utambuzi wa virusi vya ukimwi-1 kwenye damu na uwezekano wa virusi ambavyo huenda vikawa sugu kwa matibabu, kumekuwa na kupungua kwa mahitaji ya kiwango cha kadri cha vipimo vya wingi wa virusi na aina ya virusi. Matumizi mengi ya kupima wingi wa virusi na aina ya virusi katika sehemu zilizo na upungufu wa bidhaa, hairudishwi nyuma tu na gharama za juu za hivi vipimo lakini pia na ukosefu wa mtandao mzuri wa barabara. Changamoto mojawapo ni kuhakikisha ubaridi, haja ya kukusanya na kusafirisha sampuli za damu. Kwa sababu hiki kipimo chetu cha in-house genotyping kilikubaliwa kwa matumizi ya cellular DNA kutoka kwa damu nzima na matone ya damu yaliyokauka (DBS).

Utafiti wetu ulionyesha uwezekano wa kuzitenganisha kasha kupangilia DNA ya virusi vya ukimwi-1 kutoka kwa damu nzima na DBS. Ijapokuwampangilio wa DNA unaweza kusaidia katika masomo ya afya za jamii, kwa kukagua aina tofauti tofauti za virusi vya ukimwi zinavyopatikana katika jamii na usambazaji wa virusi sugu kwa matibabu, utafiti wetu ulionyesha kwamba mpangilio wa RNA bado ni bora kuliko mpangilio wa DNA katika kumfuatilizia mgonjwa mmoja mmoja, haswa mwanzoni mwa kushindwa kwa matibabu.

Kuongeza ueneaji wa ART katika nchi za chini na zile za kiwango cha kadri cha mapato imeonyosha kuwa ya mafanikio, mbali na upungufu wa vipimo vya mahabara vya kuangalia hali za wagonjwa walioko kwenye matibabu. Pia, tuliona mafanikio ya matibabu ya asilimia 86.2 kwa wale waliotibiwa kwa muda usiopungua miezi 6 katika kituo cha utunzaji wa kina (comprehensive care center) cha Mombasa. Hata hivyo kiwango cha ulumbikizaji wa virusi sugu miongoni mwa wagonjwa ambao matibabu hushinda ni jambo la kutia shaka. Katika utafiti wetu, asilimia 87.5 ya wagonjwa walio na kiwango cha virusi kinachoweza kutambulika, walipata usugu dhidi ya transcriptase inhibitors. Kinachotia shaka zaidi ni kwamba asilimia 62.5 ya wagonjwa walioshindwa na matibabu, walijenga usugu dhidi ya baadhi ya madawa. Hii itakuwa na athari kubwa katika mipango ya baadaye ya matibabu, haswa kwa sababu ni madawa machache tu ya matibabu ya mkondo wa pili yanapatikana katika sehemu zenye uhaba wa bidhaa. Upimaji wa mara kwa mara wa kiwango cha virusi unaweza kuepusha ulumbikizaji wa virusi vilivyo sugu kwa matibabu, na kukiwemo ushauri wa kuendeleza matumizi ya dawa, yanaweza kuongeza uwezekano wa kubaki katika kiwango cha kwanza cha matibabu kwa muda mrefu.

Juhudi za ulimwengu za kufanya ART zifikiwe na watu wote lazima ziendelee, mbali na kuwepo kwa uwezekano mdogo wa kuwaangalia wagonjwa walio katika matibabu kwenye sehemu za mapato ya chini na ya kadri. Hata hivyo, haja ya kuwa na vipimo vya kiwango cha virusi na usugu kwa bei nafuu na vinavyo aminika ni lazima kutiliwe maanani. Watafiti na kampuni za biashara ni lazima waendelee kutafiti vipimo vya mahabara vyenye bei nafuu ili kuwafuatilizia wagonjwa wa virusi vya ukimwi-1 walio kwenye matibabu na madawa zaidi ya ARV (generic) pia yawe yanaweza kupatikana katika sehemu zenye mapato ya chini ili kuboresha utunzaji wa walio na virusi vya ukimwi ulimwenguni.

Chapter I: Introduction and research objectives

1.1. History of Human Immunodeficiency Virus (HIV)

Promising progress has been made in recent years to tackle the HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) epidemic. This has included increasing access to effective treatment for those already infected, and prevention programmes to control the spread of the epidemic. However, the number of people living with HIV continues to grow, and it is estimated that currently, about 33 million people and their families are affected by HIV [1].

Symptoms of AIDS were first recognized in 1981, when Gottlieb *et al.* reported an increasing number of cases with Karposi's Sarcoma (KS) and *Pneumocystis Carinii* Pneumonia (PCP) in New York [2]. It was only in 1983 that HIV was recognized as the causing agent of AIDS [3, 4].

1.2. HIV virology

1.2.1. HIV structure and genome

HIV belongs to the group of lentiviruses in the family of Retroviridae, which are enveloped, positive-stranded RNA viruses. Following infection with the virus, the RNA genome is transcribed into double-stranded DNA with the viral enzyme reverse transcriptase. The produced double-stranded DNA is subsequently integrated into the host genome [5, 6].

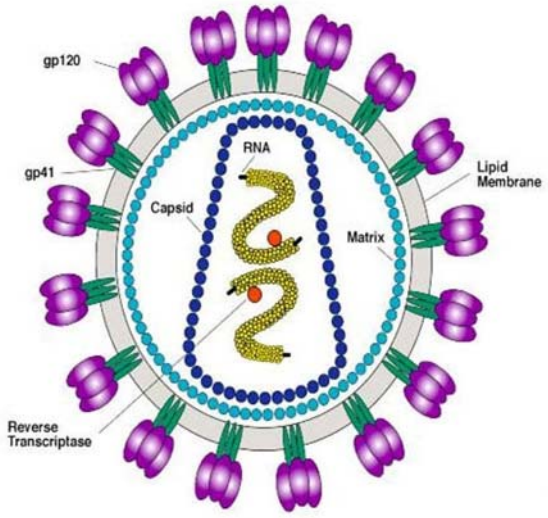


Figure 1: HIV virion structure

Adapted from : www3.niaid.nih.gov

HIV virions are spherically shaped with a diameter of 110 nm and consist of a lipid bilayer membrane or envelope, surrounding a cone-shaped nucleocapsid (figure 1). The envelope is derived from the host cell plasma membrane and is dotted with approximately 72 spikes. Each spike is composed of surface units (gp120, SU) that are non-covalently linked with the transmembrane units (gp41, TM). The inner surface of the lipid bilayer is lined with matrix proteins (MA). The capsid protein p24 forms the nucleocapsid (NC) that packs the two single stranded RNA copies with the reverse transcriptase (RT) molecules. Other viral enzymes, protease (PR) and integrase (IN) and some accessory and regulatory proteins (p6, *Nef, Vif and Vpr*) are also found in the nucleocapsid [5, 7].

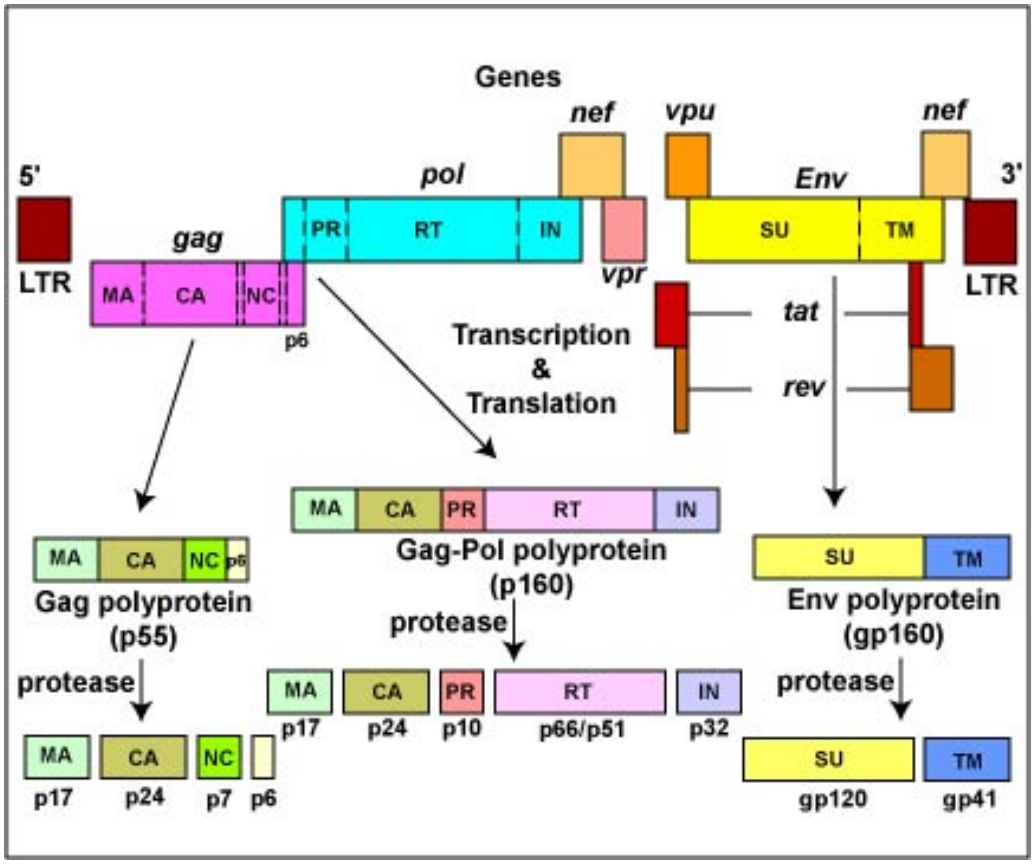


Figure 2: Genomic structure of the human immunodeficiency virus
Adapted from: http://student.ccbcmd.edu/courses/bio141/lecguide/unit3/viruses/hivgenes.html

An HIV-virion contains two identical copies of positive, single stranded RNA of about 9.2 kb long. The HIV-1 genome contains nine open reading frames (ORFs). Three of these ORFs encode for the *Gag, Pol* and *Env* precursor polyproteins which are subsequently proteolysed into individual proteins (figure 2). The *Gag* gene encodes for the precursor of the matrix proteins (MA, p17), the capsid protein (CA, p24), the

nucleocapsid protein (NC, p7) and p6. The genes for the viral enzymes: protease (PR, p10), reverse transcriptase and RNaseH (RT, p66/51) and integrase (IN, p32) are found in the *Pol* region. The *Env* gene encodes for the precursor for the envelope glycoproteins gp120 (SU) and gp41 (TM). The HIV genome also encodes for two regulatory peptides (*Tat* and *Rev*) and four accessory peptides: *vif, vpr, vpu* and *nef*. HIV has two genomic forms, single stranded RNA in the extracellular phase of the viral replication, and double stranded DNA, integrated as provirus in the host genome. In the proviral genome, the ORFs are flanked by two long terminal repeat (LTR) regions, which are essential for integration and contain promoters for the transcription of the viral genes [6, 8].

1.2.2. Replication cycle

The HIV-replication cycle can be subdivided in a number of steps (figure 3). The infection of a target cell by the virion is initiated by an interaction between the extra-cellular domain of the viral envelope glycoprotein (gp120) and the CD4 receptor located on the plasma membrane of T-lymphocytes and macrophages. This process needs additional interaction with the CCR5 or CXCR4 chemokine co-receptors, located on the cell surface (step 1). Subsequently, the transmembrane (TM) units undergo a conformational change which leads to the fusion of the viral lipid bilayer and the host cell plasma membrane whereby the viral core penetrates the cell (step 2). The virion core is then uncoated to expose the viral nucleoprotein complex consisting of MA, RT, IN, *Vpr* and RNA (step 3). After the viral genome is reverse transcribed into a double stranded DNA by viral RT (step 4), the complex is transported to the nucleus (step 5). The DNA is then integrated into the host genome, a step catalysed by the integrase enzyme (step 6). In the next step, viral transcripts are expressed from the promoter in the 5' LTR while *Tat* enhances the rate of transcription (step 7). The viral *Rev* protein regulates the processing of the RNA transcripts. Most transcripts are double spliced to generate the *Tat*, *Rev* and *Nef* proteins (step 8). When sufficient *Rev* protein is produced, it protects the viral transcripts from being spliced and mediates their transport to the cell plasma. In this way single spliced and unspliced transcripts are produced (step 9). In step 10 the single spliced transcripts are translated into *Gag, Gag-Pol* and *Env* precursors. The *Env* precursor is subsequently spliced by a cellular enzyme and gp120 and gp41 products are transported from the endoplasmic reticulum to the cellular membrane

(step 11). The *Gag* and *Gag-Pol* polyproteins, the unspliced RNA transcripts and the *Vif*, *Vpr* and *Nef* proteins are assembled into a new virion (step 12). This new virion starts to bud from the cell membrane, spiked with *Env* glycoproteins and is subsequently released from the cell (step 13). During the maturation step, the *Gag* and *Gag-Pol* polyproteins are cleaved by the viral protease, assisted by the *Vif* protein. The mature virion is now ready to infect a new cell (step 14) [6-8].

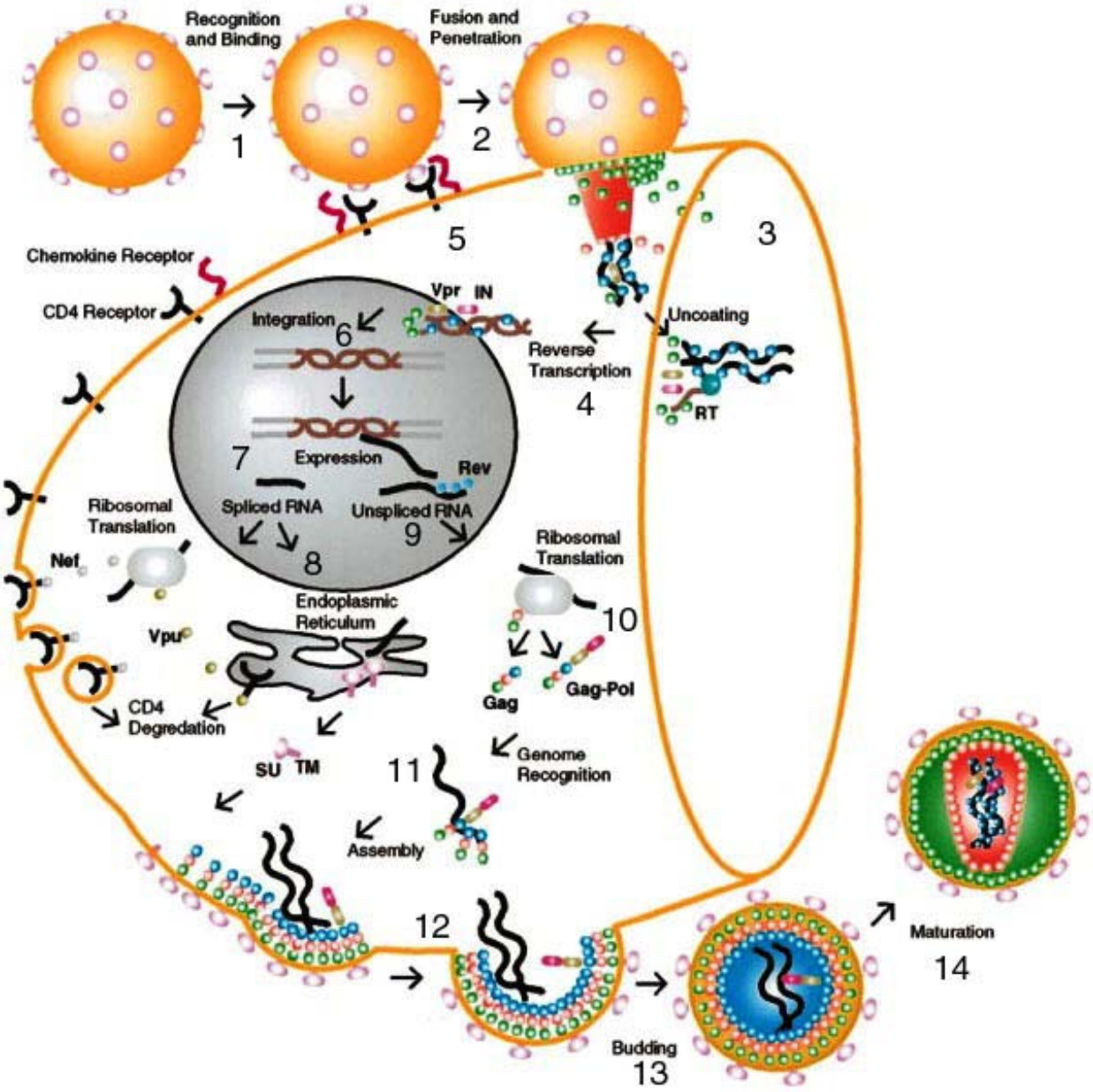


Figure 3: Replication cycle of HIV

The different steps of the replication cycle are explained in the text.

Adapted from: Turner and Summers, J Mol Biol, 1999 [7]

1.2.3. Immunopathogenesis of HIV infection

The course of HIV infection differs substantially from individual to individual. The time from initial infection until progression to AIDS in typical progressors is eight to ten years. However a subset of patients (10 to 15%) progress to AIDS within two to five years (rapid progressors), while long-term non-progressors (<5%) remain asymptomatic for at least 10 years [9].

The clinical course of HIV generally includes three phases or stages: primary infection, clinical latency and AIDS. Some HIV-infected patients remain asymptomatic during the **primary infection phase**, though most show symptoms of a flue-like syndrome including symptoms like fever, sore throat, skin rash, nausea etc. [10]. Virologically, a burst of viremia (up to 10^7 HIV RNA c/ml of plasma) and a high level of infected peripheral blood mononuclear cells (PBMCs) are associated with this primary phase of the infection [11-13], while on the immunological level, the $CD4^+$ T cells rapidly decrease. The control of virus expression by the immune response is not very effective in the period between the acute primary infection and the chronic infection. Six to eight weeks after the onset of symptoms, the acute viral syndrome resolves and the HIV specific immune responses appear. HIV specific cytotoxic T lymphocytes eliminate virus-expressing cells and production of antibodies against different HIV proteins may contribute to the down regulation of viremia [11, 12]. These antibodies form immune complexes, trap the virus in the reticulo-endothelial system, while neutralizing antibodies are only detected several months after seroconversion [14]. The **clinical latency phase** starts with the down regulation of viremia and a temporary rise of $CD4^+$ T lymphocytes. Due to the lack of ability to completely regenerate or repopulate the $CD4^+$ T lymphocytes, a persistent decline in the immune system is observed, while virus replication is still ongoing at the baseline level. When the $CD4^+$ T cell count drops < 200 cells/µl the immune system is no longer capable of controlling the virus and other pathogens and the patient becomes vulnerable to opportunistic infections. This is the onset of the **AIDS** [9]. The evolution of the HIV infection is depicted in figure 4.

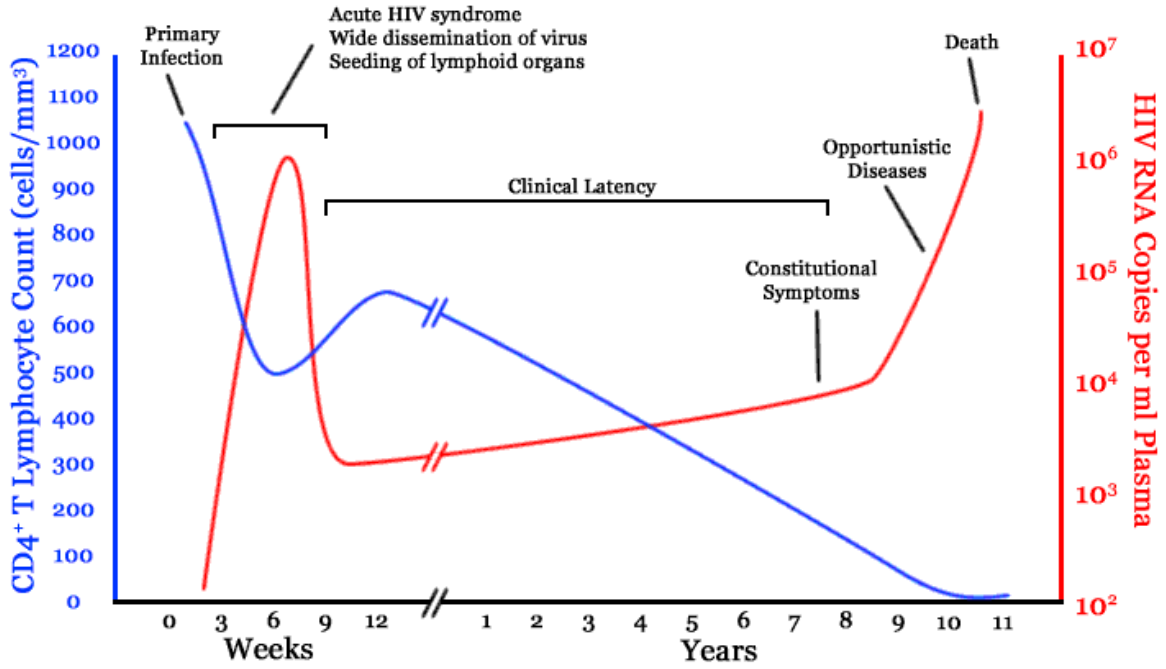


Figure 4: The natural evolution of HIV infection

Adapted from: Fauci et al. Ann Intern Med, 1996 [15]

1.3. HIV diversity and geographical distribution

Different factors contribute to the extremely high genetic heterogeneity of HIV. With only 2.5 days needed to produce new viruses, the turnover of the virus is short and about 10^{10} virions are produced daily [16, 17]. The viral reverse transcriptase lacks proofreading and as a result, a high rate of incorrect nucleotide substitutions occurs (10^{-4}/nucleotide or 1 mutation per replication cycle). Moreover a recombination process makes the exchange of larger genomic fragments possible [18]. As a result the natural polymorphisms in HIV proteins is high [19, 20].

Based on different reactions in antibody binding assays and on phylogenetic analysis of genetic sequences, two types of HIV are recognized: HIV-1 and HIV-2. While HIV-1 is widely distributed throughout the world, the spread of HIV-2 is mainly limited to West-Africa [21]. HIV-2 is further divided in 5 subtypes. Nucleotide sequence comparisons allow distinction of 3 groups among HIV-1 isolates. Most HIV-1 isolates can be classified as group M viruses, while group O represents some rare outliers. In 1998, a third group (group N) was proposed to classify HIV-1 isolates that were different from group M and O viruses. Within group M, nine subtypes (A-D, F-H, J and K) and 32 circulating recombinant forms (CRFs) are recognized [22]. The global HIV-1 subtype distribution is shown in figure 5. Subtype C is the most widespread of all HIV-1 infections (50%) while subtype A, B, D and G are responsible for 12, 10, 3

and 6% of the infections, respectively. Subtypes F, H, J and K together cause only 0.94% of the infections worldwide. About 10% of the people living with HIV are infected with CRF01_AE (5%) or CRF02_AG (5%), the two most important CRFs. Other recombinant forms account for 8% of the remaining infections. [23]

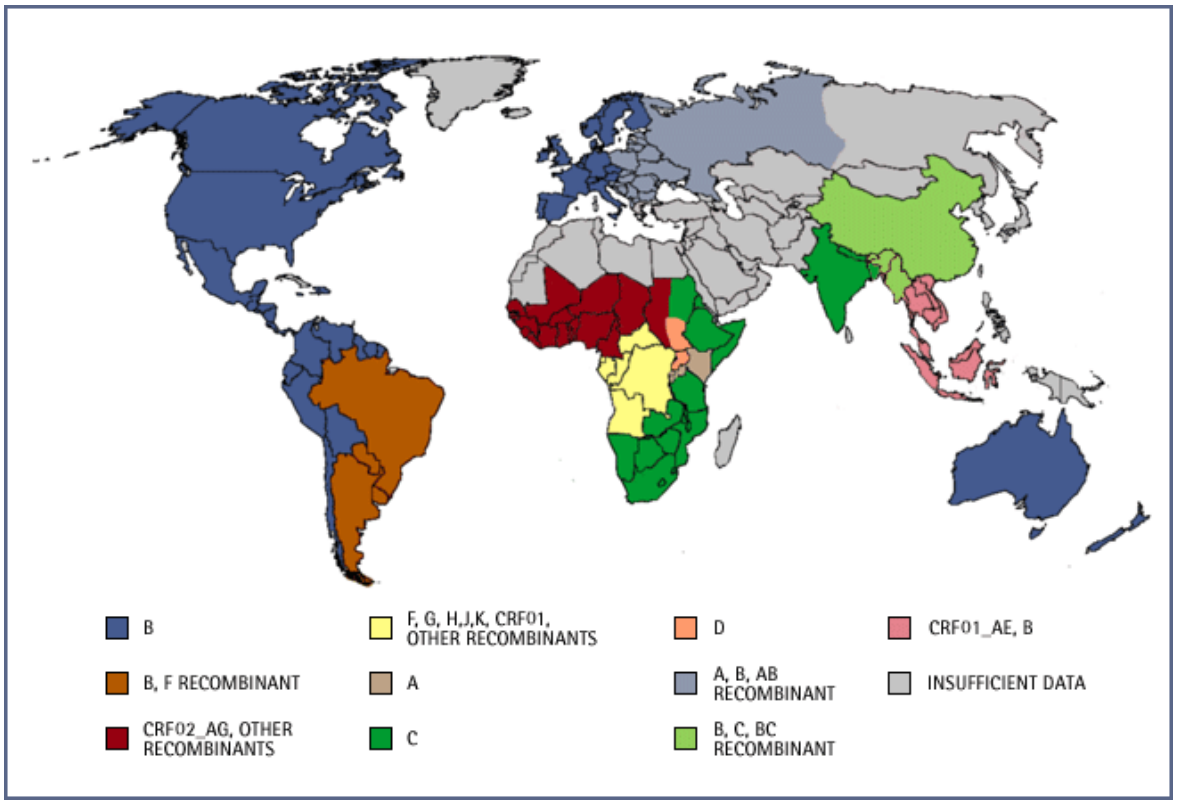


Figure 5: Regional distribution of HIV-1 subtypes and recombinants
Adapted from: http://www.pbs.org/wgbh/pages/frontline/aids/atlas/clade.html

When we look at the subtype distribution, we see that subtype C is almost exclusively responsible for all infections in Southern Africa, India and Ethiopia, responsible for 30, 13 and 4% of global infections respectively. In West Africa, which harbours 16% of the global HIV-1 infections, subtype A, G and CRF02_AG are the most predominant. Subtype A, C, D and unique recombinant forms make up most of the HIV-1 infections in East Africa, which has 10% of global infections. Subtype A dominates the subtype diversity in Kenya, while subtypes D, C and G are responsible for most of the remaining infections [24]. The greatest subtype diversity is detected in Central Africa, which contributes to only 5% of patients globally infected with HIV-1 live. CRF01_AE is responsible for most infections in South and South-East Asia (excluding India), while in East Asia the burden of infections is caused by subtype B, CRF01_AE and other recombinants. In North Africa and the Middle East subtype C

and D predominate. Subtype A and B are responsible for most HIV-1 infections in East-Europe and Central Asia and CRF03_AB has only been identified in individuals from Eastern European origin. Finally subtype B is the abundant subtype in North America, West-Europe, the Caribbean and Latin America.

1.4. Antiretroviral therapy and resistance

A first breakthrough in antiretroviral therapy (ART) was made in 1987 with the approval of zidovudine (AZT) by the US Food and Drug administration (FDA). Though the first reports proved efficacy of the drug, it was soon discovered that HIV quickly developed drug resistance when AZT monotherapy was given [25, 26]. During the next couple of years other RT inhibitors became available, but it was only in 1996 that a major breakthrough trial was announced at the 3rd Conference on Retroviruses and Opportunistic Infections where Gulick *et al.* showed the potent antiretroviral activity of the triple combination of two RT inhibitors and a protease inhibitor [27]. Soon thereafter some publications showed that what is now generally accepted as triple therapy, also known as highly active antiretroviral therapy (HAART), can suppress viral load to levels below the detection limit of current assays and restore the number of $CD4^+$ T cells in peripheral blood close to normal levels [28, 29]. HAART was quickly introduced in the clinic and had a dramatic impact on the mortality and morbidity among HIV-infected patients. By October 2007, 24 antiretroviral drugs had been approved by the FDA. These can be divided in five classes (table 1).

NRTIs	NNRTIs	PIs	Fusion and Entry Inhibitirs	Integrase Inhibitors
Abacavir (ABC)	Delavirdine (DLV)	Amprenavir (APV)	Enfuvirtide (ENF)	Raltegravir (RAL)
Didanosine (ddI)	Efavirenz (EFV)	Atazanavir (ATV)	Maraviroc (MVC)	
Emtricitabine	Nevirapine (NVP)	Darunavir (DRV)		
Lamivudine (3TC)		Fosamprenavir (APV)		
Stavudine (d4T)		Indinavir (IDV)		
Tenofovir (TDF)		Lopinavir (LPV)		
Zalcitabine (ddC)		Nelfinavir (NFV)		
Zidovudine (AZT)		Ritonavir (RTV)		
		Saquinavir (SQV)		
		Tipranavir (TPV)		

Table 1: FDA approved antiretroviral drugs

Adapted from http://www.fda.gov/oashi/aids/virals.html, October 2007

Though it is proven that HIV drug resistance emerges much slower under the pressure of a combination therapy, resistance mutations against all drug classes have been reported.

Nucleoside/nucleotide RT inhibitors (NRTIs) compete with the natural dNTPs to bind the RT enzyme. Once a NRTI is incorporated in the DNA, it acts as a chain terminator because it lacks a 3' hydroxyl group. Resistance against this class of RT inhibitors can arise either by amino acid changes that increase the discrimination between natural nucleotides and NRTIs or by promoting the removal of NRTIs [30].

Unlike NRTIs, **non-nucleoside RT inhibitors** (NNRTIs) do not need activation by intracellular kinases. NNRTIs cause an inactivating conformational change in the RT enzyme by binding the hydrophobic pocket. These inhibitors are HIV-1 specific and will therefore not bind HIV-2 RT or cellular DNA polymerase. Some mutations in the RT gene cause resistance by altering size, shape or polarity of the binding pocket or by affecting accessibility of NNRTIs to this site [31, 32].

The third class of ARV drugs, the **protease inhibitors** (PIs), bind to the PR cleavage site and inhibit the enzyme using two different mechanisms. They can bind to the active site, or mimic the transition state during peptide cleaving. Unfortunately, the hypothesis that the PR gene does not mutate as readily as RT gene has not been proven. On the contrary, polymorphisms have been found at more than half of the amino acids of PR. Additionally, more than 20 PR mutations are associated with reduced susceptibility to PI treatment. Major PI mutations can cause a conformational change by a single point mutation, thereby improving the selection of natural substrates for the protease enzyme. A number of minor mutations, in combination with a primary mutation, result in high level resistance due to the compensation for the loss of viral fitness of a mutated virus by the secondary mutations. Moreover, some mutations in the *gag* gene can adapt the *gag* cleavage site or improve the incorporation of PR in the virion in order to optimize the cleavage activity of a mutated PR enzyme [33, 34].

Lately 3 new drug classes have been added to the list of FDA approved ARV drugs. **Fusion and entry inhibitors** work by preventing the virus from infecting a cell, by either inhibiting fusion between the virus and the host cell or by blocking the co-receptors. Only one fusion inhibitor (ENF) was has been approved by the FDA. It is the binding of gp120 to the cellular receptor CD4 and a chemokine co-receptor, triggering a series of complex conformational changes in gp41 that leads to the

fusion of viral and cellular membranes. ENF inhibits this fusion by preventing gp41 to undergo a fusogenic conformation. Unfortunately, HIV can again reduce potency of this drug by the selection of resistance mutations. Recently, the first co-receptor blocker, Maraviroc, was approved by the FDA. This drug makes the CCR5 receptor unavailable for 'R5 tropic' HIV, whereby the virus cannot engage with the $CD4^+$ T-cell to initiate an infection. The last new class of antiretroviral drugs that has received a lot of attention lately are the **integrase inhibitors**. Raltegravir, which was approved by FDA in October 2007, prevents the integration of the viral genome into the host cell DNA.

1.5. Clinical assessments and laboratory tests in HIV-infected patients

After an HIV-infection has been confirmed by an HIV antibody test, clinical staging is part of the baseline assessment on entry into care and treatment programs. Additionally two surrogate laboratory markers, the number of $CD4^+$ T cells in the blood and the concentration of HIV RNA in the plasma (viral load), are routinely used to determine disease progression, the need for treatment and the efficacy of drug regimens.

1.5.1. Clinical staging

The WHO HIV clinical staging system [35] remains an essential and cheap tool to assess the disease status at baseline, to guide the decision to start ART and to follow-up patients on treatment. The disease progression is divided into four stages. The first one is the asymptomatic stage. Patients with mild clinical symptoms like herpes zoster infection, recurrent oral ulcerations, chronic upper respiratory tract infections among others, are classified as being in clinical stage 2. In clinical stage 3 symptoms become more advanced and chronic diarrhoea, unexplained persistent fever, TB and chronic lung disease etc. can appear. Finally, the occurrence of Kaposi's Sarcoma, pneumocystis pneumonia, severe wasting, chronic herpes simplex infections etc. point to the manifestation of the AIDS or clinical stage 4 (table 2). This staging system is quite sensitive in predicting disease progression and treatment failure when combined with an immunological staging or even viral load assays, when available.

WHO Clinical stage	HIV associated symptoms	Immune Status ($CD4^+$ T cells/µl)
1	no symptoms	>500
2	mild symptoms	350-499
3	advanced symptoms	200-349
4	severe symptoms	<200

Table 2: WHO Clinical staging

1.5.2. $CD4^+$ T cell testing

The European [36, 37] and US guidelines [38] recommend a baseline $CD4^+$ T cell count and a viral load test at the first visit, after HIV infection has been confirmed by an antibody test. The $CD4^+$ T cell count serves as a clinical indicator of the immunocompetence of the HIV-infected individual. The number of $CD4^+$ T cells is the most important consideration in the decision of ART initiation. In the developed world it is generally recommended to start treatment as soon as the $CD4^+$ T cells drop below 350 cells/µl [36-38], while WHO recommends to start treatment in resource-limited settings before the $CD4^+$ T cells drop below 200 cells/µl [39]. An increase in $CD4^+$ T cells of 100 cells/µl per year is an indication of adequate viral suppression on treatment [40]. Ideally, the $CD4^+$ T cell count should be performed at least every 3 to 6 months.

1.5.3. Viral load testing

Plasma HIV RNA viral load is a critical parameter for evaluating response to ART. The goal of ART is to suppress the viral replication to a level that viral RNA is no longer detectable in the plasma. Effective regimens and good adherence should result in a viral load decrease of at least 1 $\log_{10}$ c/ml per month and the achievement of an undetectable viral RNA level (< 50 c/ml) within 16 to 24 weeks [41]. It is therefore recommended that the viral load should be monitored closely after treatment initiation until full viral suppression is obtained [36-38]. Once viral load suppression has been obtained, guidelines recommend a regular viral load follow-up every 3 to 6 months to confirm that the virus remains suppressed [42, 43]. A confirmed rebound in plasma viral load may indicate a poor adherence, the development of drug resistance hence virological failure, or both [44].

The inability to detect virological failure, due to unavailability of viral load assays as is often the case in resource-limited settings, may lead to an accumulation of resistant mutations and the selection of viruses with broad cross-resistance to antiretrovirals. This might jeopardize future treatment options for the individual patients and for recently infected patients who could be infected with a circulating resistant virus.

1.5.4. Resistance testing

When clinical, immunological or virological parameters indicate that a specific regimen does not have the desired effect of viral suppression, a resistance test can provide information about the presence of resistance mutations. This information can help the clinician to choose the most appropriate new regimen. The limited experience with mono- and bi-therapy in resource-limited settings might restrict the prevalence of resistant virus in these regions compared to Europe and the US where broad scale use of suboptimal NRTI regimens was standard practice before the introduction of the first PI in 1996. However, the development of resistance is inevitable. When a first-line regimen is failing, resistance testing is not strictly necessary if all drugs in the regimen can be replaced by drugs without possible cross-resistance with the drugs in the failing regimen. Ideally drugs from a different class are chosen. Resistance testing in developing countries however might be indicative from the first failure onwards because alternatives for a second-line treatment are limited and drugs from a different class are not always available. Resistance test guidance of this second regimen can improve the efficiency. Resistance testing becomes more useful when a second-line regimen is failing because it is more difficult to find drugs without possible cross-resistance. A salvage or third-line regimen cannot be chosen without information about the specific resistance mutations in the patients' virus.

Moreover, resistance testing can be a useful tool to prevent unnecessary treatment switches in patients with an extremely low adherence. In these patients we might find virological failure while there is no evidence for resistance due to the low intake of ARVs. In this situation a switch to an expensive second-line regimen can be prevented by focusing on the improvement of the patient's adherence [45, 46].

Resistance testing may also be valuable in selecting the initial treatment regimen in regions were transmitted drug resistance (TDR) is an increasing concern. The prevalence of TDR is significant in countries with established ART programs. Though it is difficult to compare data from different studies, the highest prevalence of TDR is

seen in the US and Western Europe (6.0% to 24.1%) and some parts of South-America (3.1% to 22.2%). Since ART became more readily available in resource-limited settings, a low TDR prevalence has also been detected in some Sub-Saharan countries [47]. These regions are even more at risk because therapy switches are often based on clinical criteria allowing a prolonged virus replication under drug pressure and thereby promoting the development of drug resistant strains. However, an excellent virological and immunological response is seen in TDR patients when the first-line regimen was based on the baseline resistance results [48, 49]. Resistance testing is not only useful before initiation of treatment at an individual patient level, it can also be used in population based studies to monitor the success of treatment initiatives and the effectiveness of HIV transmission prevention programs among HAART treated patients [50].

1.5.4.1. Phenotypic resistance testing

Phenotypic assays measure the susceptibility of the virus to the drug directly in an in vitro culture assay. This was done initially by enzyme-based assays [51, 52], but because of the high cost and the labour-intensity of the procedure a new, replication-based assay, was developed. The PCR-amplified gene of interest is incorporated in a HIV backbone or vector [53-56]. In these assays the susceptibility of the viruses to antiretroviral drugs can be measured by the fold difference in drug concentration that is needed to inhibit the vector containing the patient sample compared to the wild-type vector. Results are expressed as fold change in IC_{50} whereby the IC_{50} is the drug concentration needed to inhibit 50% of the virus replication.

Even though the interpretation of phenotypic tests might seem less complicated, because the susceptibility of the virus is directly measured, an expert opinion is still needed as ‘clinical cut-offs’ are scarcely available. Clinical cut-off values are based on clinical observations from various drug resistant patients in clinical trials and cohorts. At the moment clinicians usually receive two cut-off values: the lower cut-off value that gives an idea of when the susceptibility begins to decline, but the drug still has partial activity; and the upper cut-off value that is the fold change at which the drug completely looses all activity. Because clinical cut-offs are only available for a limited number of drugs, the clinician depends on ‘biological cut-offs’ that are derived from *in vitro* susceptibility experiments with clinical isolates from drug naïve patients. These values cannot directly link *in vitro* resistance information with the actual phenotype of the virus [57].

1.5.4.2. Genotypic resistance testing

A genotypic resistance assay determines mutations in PR, RT and gp41 at amino acid positions that are known to be related with a decreased susceptibility to antiretroviral drugs. A list of detected mutations is generated and frequently updated [58]. Mutations are represented by a code, e.g. K103N, whereby the number refers to the amino acid position in the respective genes, the first letter is the amino acid present in the wildtype and the letter at the end is the mutated amino acid.

Genotypic assays are the most commonly used methods of testing drug resistance because of their relatively low cost (at least in the industrialized world) and low turn-around time. Nevertheless, these assays have the disadvantage of just producing a list of mutations. A correlation between a single mutation and resistance towards a specific drug is not always straightforward. Characteristics such as hyper-susceptibility, cross-resistance and resensitisation are difficult to take into account when interpreting the results of genotypic assays [59]. Different algorithms for the interpretation of mutational patterns have been developed [60]. Most commonly used are the Agence National de Recherche sur le SIDA (ANRS) algorithm [61, 62], the Rega Institute (Rega) algorithm [63] and the HIV RT and Protease Sequence Database (HIVDB) algorithm (http://hivdb.stanford.edu/index.html). The first two algorithms are based on a set of rules that describe specific mutational patterns, whereas the HIVDB gives a score to each of the observed mutations. The last approach allows taking of unique or complex patterns of mutations into account. Mutation scores are based on published literature linking mutations with specific antiretroviral drugs and include correlations between the genotype and treatment history, genotype and phenotype, and genotype and clinical outcome. Mutations known to contribute to hyper-susceptibility are given a negative score. Based on the final sum of scores, the virus is said to be susceptible, potential low-level resistant, low-level resistant, intermediate resistant or high level resistant. Due to the different rules used in the various systems, results from the available algorithms to interpret the drug related mutations are not always concordant [64].

Currently two major techniques are used to detect drug resistance related mutations: sequencing assays and single point mutation assays. **Dideoxynucleotide sequencing** is a viral population-based sequencing method that determines the genetic code of the PCR-amplified target gene, based on the incorporation of

fluorescently labelled dideoxynucleotides. This method is most commonly used because it has no limitations on the number of mutations that can be detected. However, it is less sensitive for minor variants, as each variant should present at least 25% of the virus population to become detectable [65]. Several sequencing methods are commercially available, though a number of laboratories have developed their own in-house methods to reduce costs.

The **single point mutation assays** are more sensitive for minor variants, but the variability around the codon of interest can decrease hybridization of primers and probes that are needed to detect specific mutations [66]. The biggest disadvantage of these tests is the limited number of mutations that can be detected [67] and the fact that new probes should be designed every time a new relevant mutation is discovered. Different techniques can be used to detect single point mutations. A *selective PCR* is done by performing two separate PCR reactions, one to amplify the wild-type sequence and the other to amplify the mutant sequence. The primers are specifically designed to hybridize with either the mutant or wild-type sequence. Amplification will only take place when the primer perfectly matches the target sequence [68]. In some studies, selective PCR for specific mutations were developed to detect mutations in minor populations [69, 70].

In the *oligonucleotide ligation assay* two differently labeled oligonucleotides are hybridized on a template. Only the oligonucleotide with the perfect match can be detected [66, 67, 71]. Several groups showed that this assay is more sensitive compared to population sequencing assays [72, 73], also for non-B subtypes [74].

1.6. HIV/AIDS and access to treatment in resource-limited countries

Despite the worldwide efforts to address the AIDS epidemic, the number of people living with HIV and the number of deaths due to AIDS continues to rise. At the end of 2007, about 33.2 million people were living with HIV, including 2.5 million newly infected adults and children. Unfortunately, resource-limited settings continue to carry the burden of the epidemic with 63% (24.7 million) and 20% (7.8 million) of all HIV-infected adults and children living in Sub-Saharan Africa and South/South-East Asia respectively (figure 6) [1].

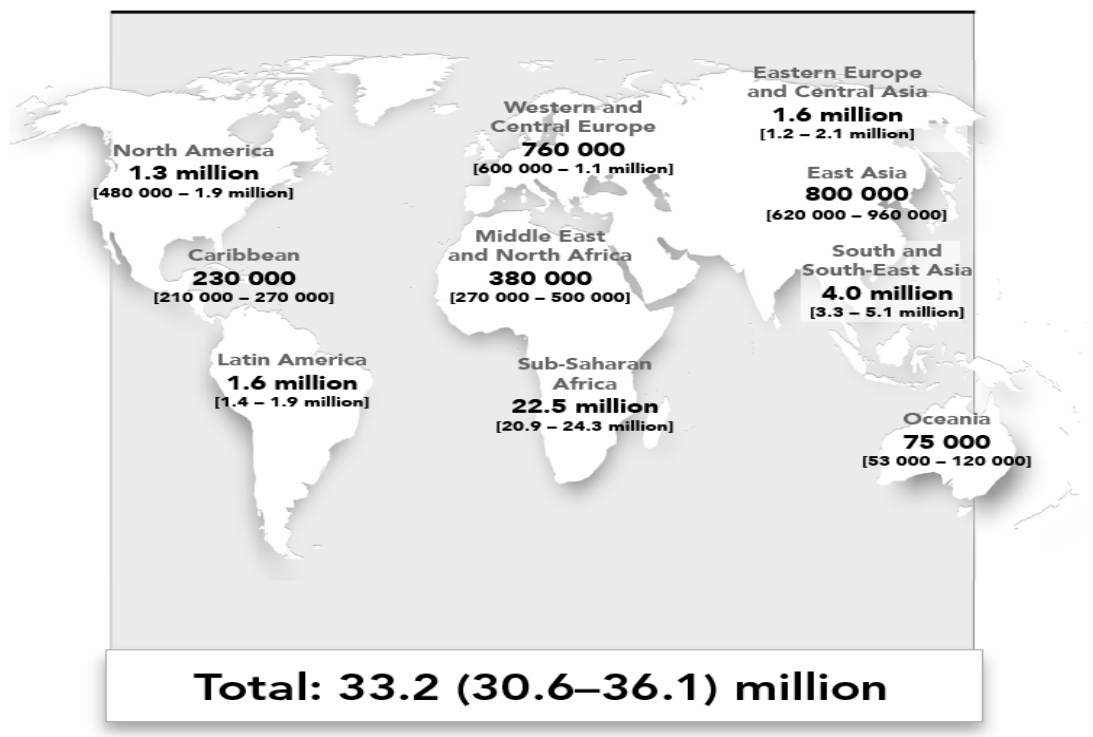


Figure 6: Adults and children estimated to be living with HIV in 2007
Adapted from: www.unaids.org

1.6.1. Scaling up access to antiretroviral therapy

Access to ART in low and middle-income countries has expanded significantly in the last couple of years. Only 400 000 people in these countries received ART at the time when WHO and UNAIDS launched the “3 by 5” initiative (December 2003). By the end of 2006, the coverage of people in need of ART in low and middle-income countries increased from 7% to 28% (to more than 2 million people). The increase was most dramatic in sub-Saharan Africa (more than 10-fold) resulting in more than 1.3 million people with access to ART today [75]. Figure 7 shows the rapid scale up of ART in Africa from 2002 to 2005, but also indicates the differences in progress between countries.

Though the pharmaceutical industry is making efforts to reduce prices for ART and increase the availability of generic products, drugs for second-line treatment are still scarce in resource-limited settings compared to the Western world [76].

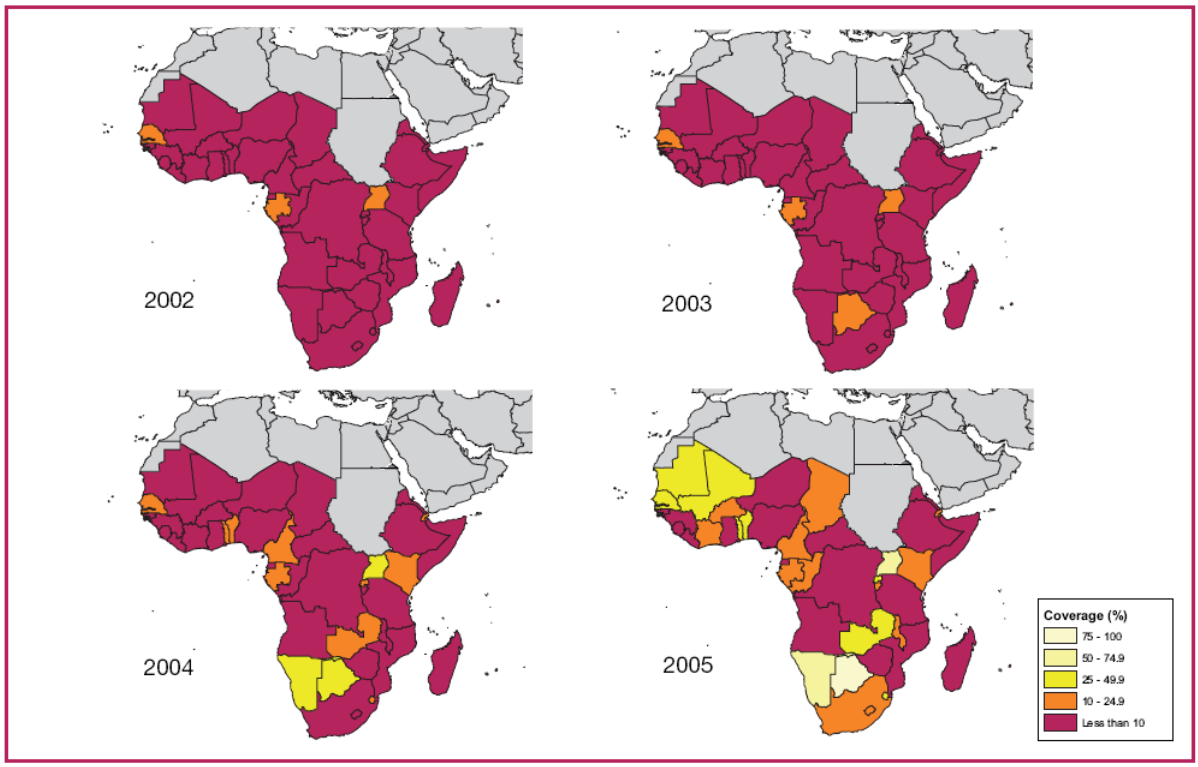


Figure 7: People in sub-Saharan Africa receiving ART as a percentage of those in need, 2002-2005
Adapted from: http://www.who.int/hiv/fullreport_en_highres.pdf

1.6.2. Scaling up access to laboratory tools to monitor patients on ART

Unfortunately, laboratory tools necessary to monitor patients on ART, are lagging behind. Traditionally, more financial resources are made available for the prevention and treatment of diseases, while almost no funding is available to improve laboratory capacity in order to ameliorate the services needed to monitor these patients [77].

Viral load tests, $CD4^+$ T cell counts and even resistance tests are considered standard of care in the Western world [36-38]. These assays can help the clinicians to decide when to start ART and when to switch a failing ART regimen to a salvage regimen. However, in some resource-limited settings, the combined cost of a commercial viral load assay and a $CD4^+$ T cell count test exceeds twice the cost of one month ART [78].

According to the WHO guidelines to scale up ART in resource-limited countries, a $CD4^+$ T cell count is desirable at baseline and every 6 months when on treatment [39]. A total lymphocyte count (TLC), possibly combined with the haemoglobin level, has been suggested as an alternative for a $CD4^+$ T cell count. This routine test has been shown feasible to guide decisions on when to start ART [79, 80], but the predictive value of TLC for the response to ART is doubtful [81, 82]. Fortunately, the

$CD4^+$ T cell tests are becoming more and more available although the supply of reagents remains challenging in most places. Moreover, some studies show a significant misclassification of therapeutic responses based on clinical and immunological criteria only [83-87], therefore viral load assays remain the preferred parameter to assess treatment responses.

Four commercial HIV-1 viral load assays are currently licensed by the FDA (Roche Amplicor HIV-1 Monitor, Biomérieux Nuclisens HIV-1 QT, Versant HIV-1 RNA 3.0 and Abbott RealTime HIV-1), with prices varying between 50 and 100 USD per test. Some companies have made a commitment to lower their prices for viral load assays in resource-limited settings, but the implementation of these tests remains problematic due to poor lab infrastructure and lack of trained personnel. Viral load assays are not only needed to detect treatment failure, but can also be useful to monitor patients' adherence to ART [45, 46] and to diagnose perinatal infection in children younger than 18 months [88-90]. It is therefore extremely important to develop cheap and easy alternative assays for viral quantification. Some low-cost alternative viral load assays have been developed in recent years. The p24 antigen (Ag) assay (Perkin-Elmer) was the first alternative test proposed for measuring the HIV-1 viral load [91]. Although initial results were promising [91-98], some conflicting results arose over the years [99-101]. Despite the doubtful use of this assay to monitor HIV-1 infected patients, p24 Ag determination remains a recommended assay for the diagnosis of perinatal HIV infection [102-107]. Another alternative for HIV viral load measurement is the ExavirLoad (Cavidi). A high sensitivity and specificity was found by several groups assessing this assay [94, 98, 108-113] and some showed the feasibility of this assay to monitor patients on ART treatment [98, 108, 109]. However most studies were conducted in well-equipped laboratories and in collaboration with the manufacturer. A third group of alternative viral load assays are the home-made real-time PCR assays. Lewin *et al.* were the first to describe an in-house real-time PCR assay to detect HIV-1 RNA [114] and others showed that the quantification of HIV-1 RNA by real-time PCR was feasible [115-120]. Rouet *et al*, found their in-house real-time PCR assay to be suitable for patient monitoring and paediatric diagnosis in Ivory Coast [119]. Based on these good results, a French company (Biocentric) decided to produce a Generic HIV viral load kit to quantify HIV-1 RNA. Performance data of ExavirLoad and Generic HIV viral load assays in

resource-limited settings are still limited and more studies are needed to confirm these promising results.

WHO does not recommend the use of genotypic resistance assays because their guidelines advise to change all components of the regimen in case of failure [39]. However, due to the limited availability of second-line ARV drugs in resource-limited settings, replacing all components of the first-line regimen is often impossible and recycling of drugs for a second-line regimen remains the only option. In this case, the efficiency of the second-line regimen will definitely improve if the choice of drugs can be guided by resistance testing. The need for drug resistance assays will increase even more when the number of available ARV drugs in resource-limited settings increases and third-line regimens become an option. The lack of viral load determinations as a parameter for early treatment failure, will increase the risk of accumulating resistance mutations leading to possible cross-resistance. This does not only pose a problem to the individual patient, but also increases the risk of transmission of highly resistant viruses in the population. Efforts to monitor the spread of resistant virus are therefore necessary. At this moment, the laboratories are not equipped to perform such high-skilled tests and the patients and health care systems lack the financial means to pay for these tests. However, at this moment it would be unethical to recommend resistance testing for individual patient follow-up as most patients don't even have access to viral load assays. Therefore WHO has initiated an HIV Drug Resistance Surveillance Program that can signal when the transmitted HIV drug resistance requires action in a particular region. The obtained information can be used to assess whether the country's standard ART regimens will continue to be effective, to evaluate the effects of post and pre-exposure prophylaxis and to give information about resistance to researchers who are developing prevention methods such as vaccines and microbicides.

Besides the technical, logistical and economical obstacles to implement treatment monitoring assays in resource-limited countries, sample collection, transportation and storage remain another challenge. Many sites lack a stable electricity supply, whereby the use of centrifuges, freezers and ACs is hampered. Due to these constraints it is often difficult obtain high quality samples that were appropriately processed and stored. In this respect the dried blood spots (DBS) are an ideal medium to collect samples in the field. This type of specimens is easy to collect by a simple finger prick, they can be air-dried and stored at room temperature until

transportation to a central laboratory. Also transportation of these samples is simplified because no dry ice shipment is required, as they can be sent my general air-mail, without the risk of infection.

Although the expansion of global access to ART should remain a priority, affordable and simple tools to assess the response to treatment are equally important.

1.7. Description of the research site

1.7.1. Mombasa, Kenya

Kenya is an East-African country bordered by Somalia, Ethiopia, Sudan, Tanzania and Uganda (Figure 8). The country's main foreign exchange earner is tourism. This is followed closely by agricultural exports such as coffee, tea and horticultural products. Almost 50% of the 35 million Kenyans live below the poverty line of 1 US$ per day.

Mombasa is a coastal island about 500 km from the capital Nairobi. It is the second largest metropolis in Kenya, with about 500,000 inhabitants It harbours the largest port in East-Africa, not only serving Kenya, but also the East-African hinterland (Uganda, Burundi, Rwanda and the Eastern part of the Democratic Republic of Congo). Mombasa has an interesting culture mix with most inhabitants being African, Arab or Swahili and a minority of Indians and Europeans.

Figure 8: Map of Kenya

Adapted from: https://www.cia.gov/cia/publications/factbook/geos/ke.html

Although recent data indicate that the prevalence among adults living with HIV in Kenya dropped from 10% in the late nineties to 7% in 2003 [121] to just over 5% today, the prevalence in some areas is still as high as 13%. Overall 1 million Kenyans are currently living with HIV [122]. About 44% of the HIV-infected Kenyan people in need of ART receive treatment. The numbers have increased from 3 000 in 2002 to 125 000 2006 [75]. Currently all provincial hospitals and 70 district hospitals in Kenya provide comprehensive HIV care, including counseling, prevention and treatment of opportunistic infections, as well as delivery of antiretroviral treatment. The goal is to reach 75% of the people needing ART by 2010.

1.7.2. International Centre for Reproductive Health (ICRH)

The International Centre for Reproductive Health (ICRH) was established in 1994 after the International Conference on Population and Development (Cairo, 1994). ICRH is a multidisciplinary centre operating within the Faculty of Medicine and Health Sciences at the Ghent University. In addition to research and training activities, ICRH is involved in the implementation of development-related projects in Africa, Asia and Latin-America. The objective of ICRH is to improve the acceptability, accessibility and quality of sexual and reproductive health services, and integrate gender sensitive and human rights based approaches in the analyses. Since 2004, ICRH is recognized as a 'WHO Collaborating Centre for Research on Sexual and Reproductive Health'.

In 2000, ICRH Mombasa was registered as a non governmental organization (NGO) in Kenya. ICRH Mombasa is dedicated to health interventions and research for the well-being of the Kenyan population, in particular in the Coast Province. The NGO has grown from a small project-oriented organization to a full-fledged Kenyan organization with over 100 staff members. Participation of Kenyan scientists and managers in all levels is constantly on the rise. ICRH is collaborating closely with the University of Nairobi and public sector institutions in Coast Province, particularly with the Coast Province General Hospital (CPGH), the Provincial Medical Officer and the Mombasa City Council.

Activities in Mombasa involve sexually transmitted infections (STI) and HIV prevention, treatment and care, including projects on behaviour-change interventions in most-at-risk populations, prevention of mother to child transmission of HIV, and female initiated prevention methods. CPGH, the second largest public hospital in Kenya, provided the opportunity and facilities to conduct the research. Capacity

building of CPGH is provided through technical advice, regular trainings for local health staff (doctors, nurses, laboratory technologists etc.), infrastructural support (a gender based violence clinic was recently built and a new biochemistry laboratory unit has been planned), as well as collaborations in clinical trial conduct.

Samples used for this research were collected at CPGH and processed and stored in the ICRH laboratory. Other samples were sent to the AIDS Reference Laboratory in Ghent, Belgium. The AIDS Reference Laboratory is an accredited laboratory, where HIV-infected patients are being monitored. ICRH has a close collaboration with this laboratory for various of their research activities.

1.8. Research Objectives

As previously described there is an urgent need to develop simple and affordable alternatives for the current viral load and resistance assays, which can be implemented in resource-limited settings.

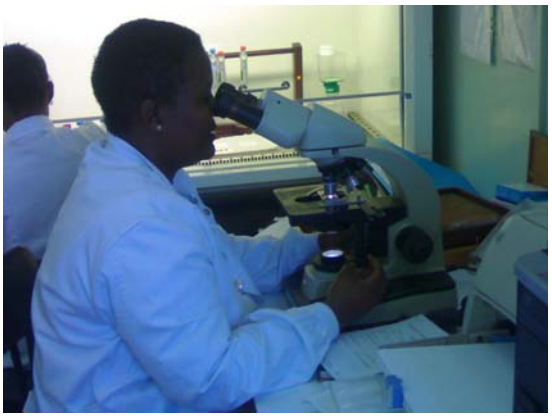
Therefore, the overall objective of this work is to evaluate existing and develop alternative methods for viral load and genotypic resistance assays, suitable for future use in resource-limited settings.

Specific objectives include:

1) to test simple and low-cost alternative viral load assays in a public hospital with limited treatment monitoring facilities
2) to study an in-house genotyping system to detect drug resistance mutations
3) to assess the feasibility of using dry blood spots for the detection of these mutations in order to simplify the sample collection and transportation process
4) to examine the treatment success and the prevalence of drug resistance among patients receiving ART at a public hospital with limited treatment monitoring facilities

In addition we attempted to strengthen the local laboratory at Coast Province General Hospital and to set-up a research laboratory where viral load assays could be performed.

Pictures ICRH



ICRHK Laboratory at Coast Province General Hospital, Mombasa

ICRHK-day at the beach

Chapter II: Results

2.1. Evaluation of two commercially available alternatives for HIV-1 viral load testing in resource-limited settings.

Kim Steegen, Stanley Luchters, Nancy Decabooter, Jacqueline Reynaerts, Kishor Mandaliya, Jean Plum, Walter Jaoko, Chris Verhofstede and Marleen Temmerman

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Evaluation of two commercially available alternatives for HIV-1 viral load testing in resource-limited settings

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Abstract

There is an urgent need for low-cost assays for HIV-1 quantitation to ensure adequate follow-up of HIV-infected patients on antiretroviral therapy (ART) in resource-limited countries. Two low-cost viral load assays are evaluated, a reverse transcriptase activity assay (ExavirLoad v2, Cavidi) and a real-time reverse transcriptase PCR assay (Generic HIV viral load, Biocentric). Both tests were compared with the ultrasensitive HIV Amplicor Monitor assay. Samples were collected in Mombasa, Kenya, from 20 HIV-1 seronegative and 150 HIV-1 seropositive individuals of whom 50 received antiretroviral treatment (ART). The ExavirLoad and the Generic HIV viral load assay were performed in a local laboratory in Mombasa, the Amplicor Monitor assay (version 1.5, Roche Diagnostics) was performed in Ghent, Belgium.

ExavirLoad and Generic HIV viral load reached a sensitivity of 98.3% and 100% and a specificity of 80.0% and 90.0%, respectively. Linear regression analyses revealed good correlations between the Amplicor Monitor and the Generic HIV viral load ($r = 0.935$, $p < 0.001$) with high accuracy (100.1%), good precision (5.5%) and a low percent similarity coefficient of variation (5.4%). Bland–Altman analysis found 95% of the samples within clinically acceptable limits of agreement (−1.19 to 0.87 log copies/ml). Although, the ExavirLoad also showed a good linear correlation with the Amplicor Monitor ($r = 0.901$, $p < 0.001$), a problem with false positive results was more significant. The cost per test remains relatively high (US$ 30 for ExavirLoad and US$ 20 for the Generic HIV viral load). Hence, false positive results and the need for an expensive PCR instrument for the Generic HIV viral load assays still limit the implementation of these tests in less equipped, less experienced laboratories.

Keywords: HIV-1; Low-cost viral load assay; Resource-limited countries

1. Introduction

Programs to scale-up ART in resource-limited countries have received a lot of attention in the last 3 years (WHO, 2006a). Currently, almost 1.5 million people in these countries are receiving ART and the numbers continue to grow. Due to limited resources and inadequate laboratory capacity, many programs have minimized the laboratory monitoring of patients on treatment, in an effort to accelerate the widespread availability of the antiretroviral drugs (Petti et al., 2006). There are, however, several reasons why equal efforts should be made to implement HIV-1 RNA viral load monitoring in these regions. The inability to detect early virological failure could lead to an accumulation of resistant mutations and the selection of viruses with broad cross resistance to antiretrovirals. An increasing number of patients carrying drug resistant virus will inevitably lead to a spread of these resistant strains in the population. Moreover, recent studies have shown that determination of the viral load, in combination

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with an adherence intervention, can assist patients in maintaining their first-line regimen, preventing unnecessary switches in treatment (Boulle et al., 2006; Reynolds et al., 2006). In addition, viral load assays, when cheaper and more user-friendly, could be used to assess ART program quality, and are a useful tool for early diagnosis of perinatally infected children (Delamare et al., 1997; Lambert et al., 2003; Rouet et al., 2001; Simonds et al., 1998).

Broadly used commercial viral load assays such as the Roche Amplicor HIV-1 Monitor, Bayer Versant HIV-1 RNA, BioMérieux Nuclisens HIV-1 QT and the Abbott real-time HIV-1 PCR assays remain too expensive (US$ 50–100 per test) (Fiscus et al., 2006; Stephenson, 2002). Recently, two more affordable viral load assays became available: the ExavirLoad assay which determines the activity of the reverse transcriptase (RT) enzyme as a marker of retroviral replication, and the Generic HIV viral load test which is a real-time PCR assay, measuring the HIV-RNA burden in plasma. The performance of these assays was compared with the Roche Amplicor HIV-1 Monitor 1.5 assay. Both alternative viral load assays were evaluated in Coast Province General Hospital in Mombasa, Kenya. The Roche Amplicor Monitor assay was performed in Ghent, Belgium.

2. Materials and methods

2.1. Patients and samples

All study participants were recruited from Coast Province General Hospital in Mombasa, the second largest public hospital in Kenya. A total of 150 adult HIV-1 infected patients attending the HIV comprehensive care centre (CCC) were selected at random. The CCC was set-up to launch the ART program in Mombasa. Of the selected patients, 50 were on ART and 100 were treatment naïve. Additionally, 20 HIV-seronegative patients were selected at random after voluntary testing and counselling at the CCC or the antenatal clinic. Written informed consent for participation in the study was obtained from all 170 study participants. The study was approved by the ethics review committee of the University of Nairobi.

The mean age of the HIV-1 seronegative subjects was 27.5 years (S.D. ± 5.9), 16 (80.0%) of them were women. The mean age of the HIV-1 infected patients was 36.9 (S.D. ± 9.2) years, 99 (66.0%) were women and 51.3% of all infected patients were in clinical stage 3 or 4 (WHO, 2006b). Median CD4 cell counts, available for 143 of the HIV-infected patients, was 243 cells/mm^3 (IQR 138–405 cells/mm^3). Patients on ART were on treatment for a mean of 13 months (ranging from 2 weeks to 33 months).

Ten millilitres of EDTA blood was collected for CD4 cell count (FACScount Becton Dickinson Immunocytometry, Oxford, UK). The remainder of the EDTA blood was centrifuged to collect the plasma which was stored in three different aliquots at −80 °C until processing. Two aliquots were used in Mombasa to perform the ExavirLoad and the Generic HIV viral load test. One aliquot was shipped to the AIDS Reference Laboratory at the Ghent University Hospital, Belgium, where the Amplicor Monitor assay (Roche Diagnostics, Basel, Switzerland) was performed.

2.2. ExavirLoad version 2 Cavidi assay

HIV-1 RT activity in plasma samples was assessed in Mombasa, using the ExavirLoad version 2 kit (Cavidi Tech AB, Uppsala, Sweden, Lot Nos. 05052 and 06018) following manufacturers' instructions. Results were evaluated using the ExavirLoad Analyser version 1.1 software. The assay measures the activity of the HIV RT enzyme in converting RNA to cDNA. A virus binding gel is added to 1 ml of plasma to purify the virus particles. After removing inhibitors such as RT inhibitor drugs and antibodies by washing steps, the viruses are lysed. The lysates are subsequently transferred to a 96-well plate for the RT activity assay. During an overnight incubation, the RT enzyme incorporates BrdUTP into a DNA strand complementary to a polyA template bound to the wells. An anti-BrdUTP antibody conjugated to alkaline phosphatase is added and the amount of incorporated BrdUTP is detected using a substrate. The colour intensity of each well is read using a standard plate reader at 405 nm. Results are extrapolated against a standard curve, expressing the HIV RT activity in fg/ml. The RT activity is automatically converted to HIV RNA copies/ml equivalents using the ExavirLoad Analyser software. The lower detection limit (LDL) of this assay is 400 copies/ml.

2.3. Generic HIV-1 viral load Biocentric assay

The Generic HIV viral load assay (Biocentric, Bandol, France, Lot Nos. 1298307 and 1401633) was also performed in Mombasa. The principle of real-time PCR is based on a fluorogenic 5′ nuclease assay: a probe with a reporter dye at the 5′-end and a quencher dye at the 3′-end is cleaved during the amplification reaction. As the reporter dye gets separated by the quencher dye, an increased fluorescence of the reporter is detected. The fluorescence is directly proportional to the initial amount of RNA present in the sample. The software produces a threshold cycle (C_t-value) from each raw fluorescence data and extrapolates that against a standard curve.

HIV-1 RNA was extracted from 200 μl of plasma using the QIAamp Viral RNA mini kit (QIAgen, GmbH Germany, Lot No. 1014795). The volumes of AVL buffer and absolute ethanol were increased as per manufacturers' instructions. Final elution was done in 60 μl of Molecular Biology Grade water (Sigma–Aldrich, Bornem, Belgium).

The Generic HIV viral load kit was used for further RT-PCR amplification. The RT-PCR targets a conserved consensus region in the long terminal repeat (LTR) region of HIV-1. Samples were first tested according to the manufacturer's instructions (referred to as 'standard method'), in a total reaction volume of 50 μl containing 20 μl of RNA, 25 μl master mix and 1 μl each of the two primers, the probe, the reference dye and the enzyme. To reduce costs, a modified version of the assay, in which the volume of all reagents was reduced by half, was also evaluated (referred to as 'modified method').

A standard curve was constructed with serial 10-fold dilutions (from 5,000,000 to 500 copies/ml) of the standard sample included in the kit. The cycling conditions were 30 min at 48 °C and 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification and data collection were carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, South Africa). The Generic HIV viral load assay has a LDL of 300 copies/ml.

2.4. Roche Amplicor HIV-1 Monitor 1.5 assay

The Ultrasensitive Cobas Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Basel, Switzerland, Lot No. H02784) with a lower detection limit of 50 copies/ml was used as a reference method and performed according to the manufacturer's instructions (Sun et al., 1998). All Cobas Amplicor Monitor analysis were run in Ghent, Belgium.

2.5. Subtyping

Protease (PR) and reverse transcriptase (RT) gene sequences were obtained using a home-made sequencing assay as described earlier (Steegen et al., 2006). Direct sequencing of both sense and antisense strands was done with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction products were analysed on an ABI310 or an ABI3130XL Genetic Analyser (Applied Biosystems). All validations and subsequent manipulations of the sequencing results as well as the interpretations of the genotyping data and the subtyping, were performed using the Smartgene™ HIV software packages (Integrated Database Network System, Smartgene, Zug, Switzerland).

2.6. Statistical analysis

All statistical analyses were performed using SPSS 15.0 (SPSS, Illinois, USA). In order to avoid bias caused by different upper and lower detection limits viral load results above 100,000 copies/ml were equalized to 100,000 copies/ml (5log copies/ml). The lower viral load values obtained with Amplicor Monitor were adapted according to the comparator assay. When the Generic HIV viral load data set was analysed, the Amplicor Monitor viral load results <300 copies/ml (LDL of Generic HIV viral load) were equalized to 300 copies/ml. When the ExavirLoad data set was analysed, all Amplicor Monitor results <400 copies/ml were equalized to 400 copies/ml (LDL of ExavirLoad). All results below the LDL of an assay, were set at half the LDL value, i.e. 150 copies/ml (log = 2.2) for Generic HIV viral load and 200 copies/ml (log = 2.3) for ExavirLoad. The normality of data distribution was examined by the Smirnov–Kolmogorov test. Direct correlations between the different data sets were measured with a two-tailed Spearman rank correlation. A Bland–Altman difference plot was generated for bias and agreement measurements, including limits of agreement (Bland and Altman, 1999). A percent similarity model was applied to determine accuracy, precision and an overall agreement between two assays (Scott et al., 2003).

Table 1
Number of patients with detectable and undetectable viral load results in the different assays

	Amplicor Monitor		ExavirLoad		Generic HIV viral load (50 μl)		Generic HIV viral load (25 μl)	
	Undetectable VL (<50 c/ml)	Detectable VL	Undetectable VL (<400)	Detectable VL	Undetectable VL (<300 c/ml)	Detectable VL	Undetectable VL (<300 c/ml)	Detectable VL
HIV negative (n = 20)	20 (100%)	0 (0%)	16 (80%)	4 (20%)	19 (95%)	1 (5%)	18 (90%)	2 (10%)
HIV positive, ART naïve (n = 100)	1 (1%)	99 (99%)	1 (1%)	99 (99%)	4 (4%)	96 (96%)	5 (5%)	95 (95%)
HIV positive, ART treated (n = 50)	31 (62%)	19 (38%)	15 (30%)	35 (70%)	35 (70%)	15 (30%)	30 (60%)	20 (40%)
Total HIV positive (n = 150)	32 (21%)	118 (79%)	16 (11%)	134 (89%)	39 (26%)	111 (74%)	35 (23%)	115 (77%)

Patients are grouped according to HIV and treatment status. VL = Viral load, c/ml = copies/ml, ART = antiretroviral therapy.

To assess a possible influence of different subtypes on the outcome of the viral load assays, an independent two tailed *t*-test was used.

3. Results

3.1. Specificity and sensitivity of the assay

Only the Amplicor Monitor assay gave an undetectable viral load result for all 20 samples from HIV-negative individuals. Positive results were obtained in one patient by the standard Generic HIV viral load (1432 copies/ml), in two patients by the modified Generic HIV viral load (673 and 724 copies/ml) and in four patients in the ExavirLoad (407, 432, 900 and 3174 copies/ml), resulting in a specificity of 95.0% (95% CI: 73.1–99.7%), 90.0% (95% CI: 66.9–98.2%) and 80.0% (95% CI: 55.7–93.4%), respectively (Table 1).

Of the 31 HIV seropositive patients on ART with an undetectable viral load in the Amplicor Monitor assay (Table 1), 30 and 27 had a viral load below the detection limit by the standard and modified Generic HIV viral load, respectively. Only 12 patients had an undetectable viral load by ExavirLoad. Of note, three out of four patients for whom a positive result was obtained with the Generic HIV viral load also scored positive in ExavirLoad. For most of the samples the viral load remained low, ranging from 413 to 3833 copies/ml in Generic HIV viral load and from 404 to 3410 copies/ml in ExavirLoad. One treatment naïve patient had an undetectable viral load in all three assays.

Of the 150 specimens from HIV-infected individuals, 118 (78.6%) had a detectable viral load in the Amplicor Monitor assay (Table 1). Of those, 111 and 113 were detectable with the standard and the modified Generic HIV viral load assay, respectively. In the ExavirLoad, 113 samples were detectable. The seven samples with a detectable viral load in the Amplicor Monitor assay that remained undetectable in the Generic HIV viral load assay, had Amplicor Monitor viral load results below the detection limit of the Generic HIV viral load assay (55, 73, 74, 81, 128, 159 and 294 copies/ml) and therefore can not be considered as false negative. The five samples with a detectable viral load in the Amplicor Monitor assay but with an undetectable result in ExaVirLoad had an Amplicor Monitor viral load of 55, 73, 74, 9790 and 15,300 copies/ml. The latter two were clearly above the detection limit of the assay and must be considered as false negative in ExaVirLoad. This results in a sensitivity of 100% (95% CI: 95.9–100%) and 98.3% (95% CI: 93.2–99.7%), for Generic HIV viral load and ExavirLoad, respectively.

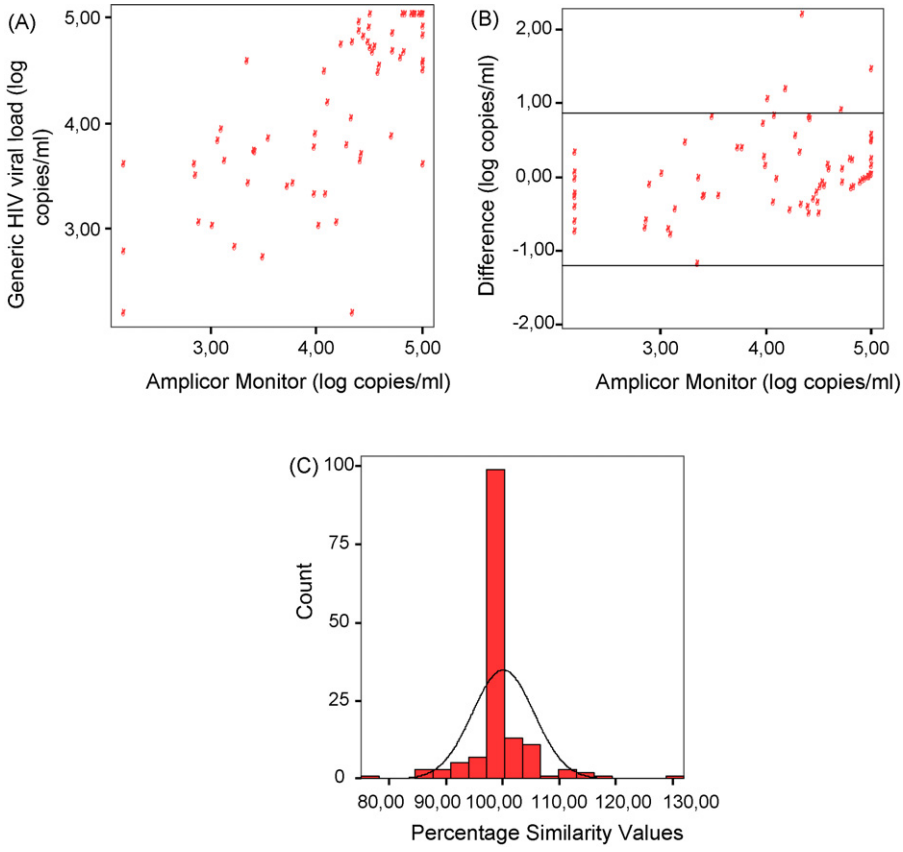

Fig. 1. Comparison between the results of the Generic HIV viral load assay (standard method) and the Amplicor Monitor assay. All samples with a viral load <300 copies/ml were equalized to 150 copies/ml (2.2log). (A) Direct assay correlation between Amplicor Monitor and Generic HIV viral load. Spearman correlation $r = 0.935$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between Amplicor Monitor and Generic HIV viral load (*Y*-axis) against the Amplicor Monitor results (*X*-axis). The bias on the difference is −0.16 (S.D.: 0.54) with limits of agreement between −1.19 and 0.87. (C) Percent similarity plot with 100.1% accuracy, 5.5% precision and a coefficient of variance of 5.4%.

Table 2
Summary of the results of the statistical analysis

	Direct assay correlation		Bland–Altman plot			Percent similarity plot		
	Spearman coefficient	*p*-Value	Bias (mean)	S.D.	Limits of agreement	Accuracy (mean)	Precision (S.D.)	Overall agreement (CV)
Generic HIV viral load (50 μl) $n = 150$	0.935	<0.001	−0.16	0.53	−1.19 to 0.87	100.1%	5.5%	5.4%
Generic HIV viral load (50 μl) $n = 63$ (trimmed)	0.835	<0.001	0.03	0.54	−1.02 to 1.08	100.3%	7.1%	7.1%
Generic HIV viral load (25 μl) $n = 150$	0.939	<0.001	0.01	0.29	−0.56 to 0.58	100.6%	4.8%	4.8%
Generic HIV viral load (25 μl) $n = 63$ (trimmed)	0.902	<0.001	0.02	0.40	−0.77 to 0.80	100.8%	6.2%	6.1%
ExavirLoad $n = 150$	0.901	<0.001	−0.03	0.51	−1.03 to 0.96	100.8%	7.8%	7.7%
ExavirLoad $n = 63$ (trimmed)	0.787	<0.001	−0.15	0.58	−1.29 to 0.98	98.9%	8.1%	8.2%

S.D.: Standard deviation, CV: coefficient of variance.

3.2. Correlation between Generic HIV viral load and Amplicor Monitor assays

Standard Generic HIV viral load results and Roche Amplicor results were available for all 150 patients. Forty-nine had a viral load >100,000 copies/ml and 31 were undetectable in both (with Amplicor Monitor LDL set at 300 copies/ml). In 40 samples a higher viral load value was found by Generic HIV viral load compared to Amplicor Monitor, whereas in 30 of the samples the Generic HIV viral load result was lower. Differences in log copies/ml were <0.5 and thus within the normal range of the test variability for most of the samples ($n = 49$). For 21 samples, the difference ranged from 0.50 to 2.16 log copies/ml. As shown in Fig. 1A, a good correlation was found between the results of both assays (Spearman correlation coefficient of $r = 0.935$; $p < 0.001$). In the trimmed data set, with exclusion of results above and below the detection limit, the correlation decreased to $r = 0.835$, but remained statistically significant. The agreement between the two assays was assessed by a Bland–Altman plot and the percent similarity model. Results are shown in Fig. 1B and C. When the modified version of Generic HIV viral load assay was compared to the Amplicor Monitor assay a comparable correlation coefficient was found ($r = 0.939$). The results of additional statistical analysis are summarized in Table 2.

3.3. Correlation between ExavirLoad and Amplicor Monitor assays

ExavirLoad and Amplicor Monitor results were available for all 150 patients. Forty-two had a viral load above 100,000 copies/ml and 16 were undetectable in both assays (with LDL Amplicor Monitor set at 400 copies/ml). In 56 samples, the difference in log copies/ml between both results was <0.5. In 11 patients the difference exceeded 1.0 log. For five of these, the viral RNA was either undetectable or above the HDL in one of the two assays. Overall, the results of both assays correlated well, with a Spearman correlation coefficient of $r = 0.901$ ($p < 0.001$) for the untrimmed data (Fig. 2A) and $r = 0.787$ ($p < 0.001$) for the trimmed data set, with exclusion of results above and below the detection limit. The results of the Bland–Altman and percent similarity plot are shown in Fig. 2B and C and in Table 2.

3.4. Correlation between Generic HIV viral load assay, standard and modified version

All samples from the 150 seropositive patients were run both with the standard Generic HIV viral load assay and a modified version with a reaction volume reduced to 25 μl. Thirty-nine and 33 samples were found with an undetectable viral load in the standard and modified assay, respectively. For 26 samples, the log difference between the viral load results of both versions was greater than 0.5, but the difference exceeded 1.0log in only 5. A good correlation was found between the results of both assay versions, with a Spearman correlation coefficient of 0.937 ($p < 0.001$) (Fig. 3A). Fig. 3B and C show the results of additional statistical analysis.

3.5. HIV-subtype distribution and influence of the HIV subtype on the results of the viral load assays

Sequencing data of the protease (PR) and (RT) gene were available for 56 out of the 60 patients for whom the sequence analysis was attempted, including all 50 patients on ART and 10 selected at random from the 100 treatment naïve patients. Proviral DNA was used for the sequencing reactions, allowing the subtyping of samples irrespective of the RNA viral load. Results revealed a subtype distribution as follows: subtype A, 55.4% ($n = 31$), D, 12.5% ($n = 7$), CRF16_AD, 12.5% ($n = 7$), C, 10.7% ($n = 6$), G, 1.8% ($n = 1$) and recombinations of mainly D, A, C and CRF_AE 7.1% ($n = 4$).

Table 3 shows the results of the three viral load assays for the 56 patients for whom a subtyping was performed. For some samples an undetectable viral load was observed in the Amplicor Monitor and the Generic HIV viral load while a positive result was obtained in ExaVirLoad. This discordance was observed more frequently in samples with a subtype D (2 out of 2) or C infection (5 out of 6), than in samples with subtype A virus (5 out of 12). An independent, two-tailed *t*-test, comparing the results of subtype A samples ($n = 31$) and non-subtype A samples ($n = 25$) showed no statistically significant subtype influence

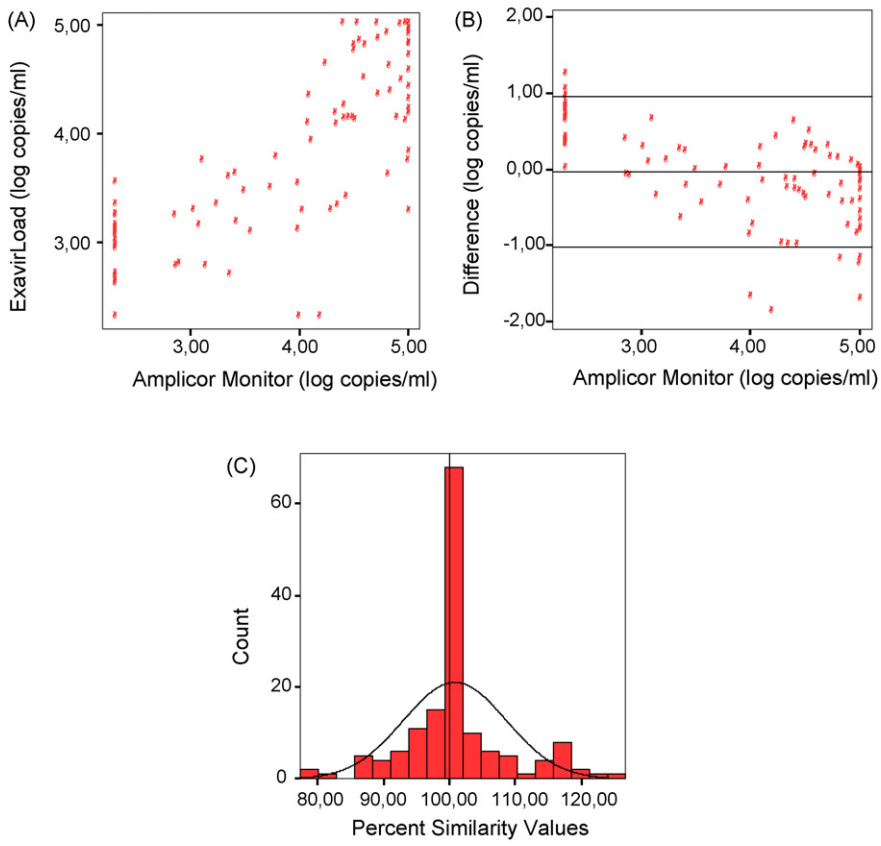

Fig. 2. Comparison between the results of the ExavirLoad assay and the Amplicor Monitor assay. All samples with a viral load <400 copies/ml were equalized to 200 copies/ml (2.3log). (A) Direct assay correlation between Amplicor Monitor and ExavirLoad. Spearman correlation $r = 0.901$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between Amplicor Monitor and ExavirLoad (Y-axis) against the Amplicor Monitor results (X-axis). The bias on the difference is −0.03 (S.D. = 0.51) with limits of agreement between −1.03 and 0.96. (C) Percent similarity plot with 100.8% accuracy, 7.8% precision and a coefficient of variance of 7.7%.

($p = 0.645$ for the Generic HIV viral load and $p = 0.699$ for the ExavirLoad).

4. Discussion

The challenge of how to appropriately monitor patients on antiretroviral treatment in resource-limited settings was only raised a few years ago with the increasing availability of ART in these regions (Majchrowicz, 2003). In Western countries, follow-up of HIV infected individuals includes at least regular CD4 cell counts and viral load quantitations, supplemented with genotypic resistance testing in case of treatment failure (CDC, 2006). Implementation of this procedure in resource-limited settings will far exceed the available budgets. In some countries, the combined cost of a CD4 cell count and a viral load test is higher than the cost of 2 months ART therapy (Stephenson, 2002). A number of alternative methods for viral load testing, including p24 antigen detection, reverse transcriptase activity testing and real-time PCR assays for viral RNA quantitation, have been evaluated by others (Fiscus et al., 2006) and good correlations were found between these assays and standard commercial viral load tests. However, most of these studies were performed in high-income countries and therefore did not take into account the possible logistical problems that might be encountered in resource-limited settings. In the study presented here, the assays were performed on-site, in the Coast Province General Hospital, a public provincial referral hospital in Mombasa. Despite the fact that all reagents had to come from overseas, delivery went fairly well, which is partly due to the fact that reagents for the assays can be bought together as a complete kit. Only the QIAgen extraction kit for the Generic HIV viral load assay had to be purchased separately.

The results of the assessed assays correlated well with the Amplicor Monitor, with Spearman correlation coefficients of $r = 0.935$ for the Generic HIV viral load and $r = 0.901$ for the ExaVirLoad. The Generic HIV viral load showed a smaller bias and narrower limits of agreement, compared to the ExavirLoad assay. Besides, the precision and overall agreement with the Amplicor Monitor was higher for the Generic HIV viral load compared to the ExavirLoad assay. Large confidence intervals for specificity of the evaluated assays compared to the Amplicor Monitor were mainly due to the small sample size of HIV-negative individuals. This must be seen as a limitation of the study.

A relatively high number of false positive results were observed, both in the small group of HIV negative individuals and in the group of patients on a successful ART regimen according to the Amplicor Monitor viral load results. Only 38.7% of

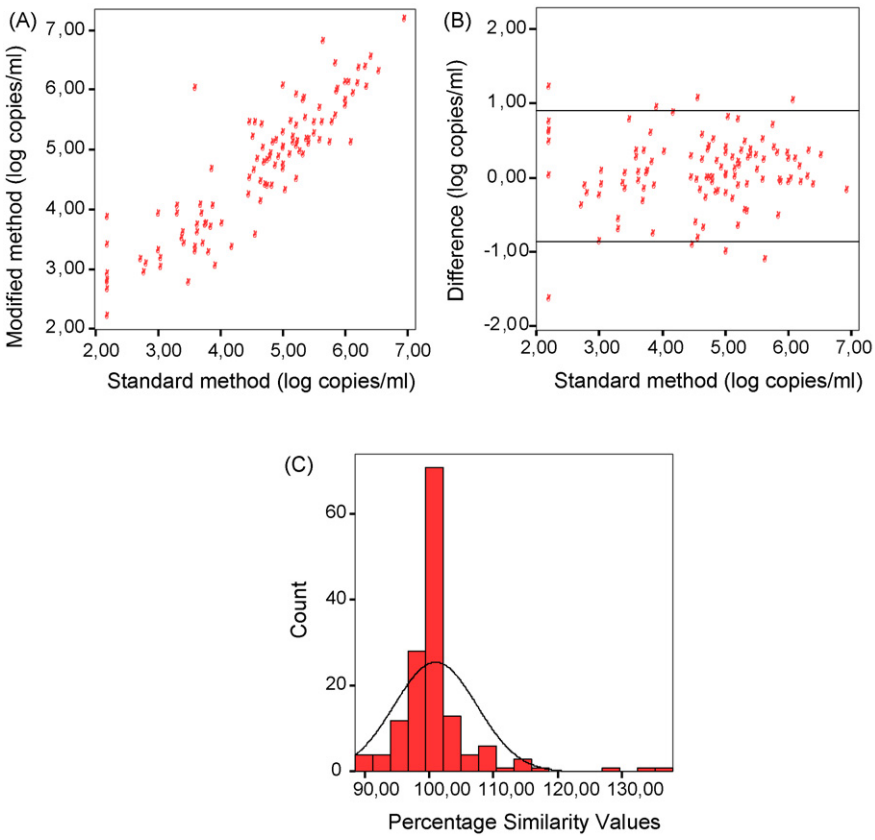

Fig. 3. Comparison between the results of the Generic HIV viral load assay, using the standard and modified method. All samples with a viral load < 300 copies/ml were equalized to 150 copies/ml (2.2log). (A) Direct assay correlation between methods 1 and 2. Spearman correlation $r = 0.937$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between the standard and modified method (Y-axis) against the results of the standard method (X-axis). The bias on the difference is 0.01 (S.D.: 0.40) with limits of agreement between −0.84 and 0.85. (C) Percent similarity plot with 100.9% accuracy, 6.5% precision and a coefficient of variance of 6.4%.

the samples from treated patients in which the viral load was <50 copies/ml in Amplicor Monitor, had an undetectable viral load in the ExavirLoad. Ninety-seven percent (96.8%) remained undetectable in the Generic HIV viral load. Further analysis of the specificity of both tests and especially of the ExavirLoad assay, on a larger number of HIV negative and successfully treated individuals, is needed. False positive results were low viral loads in the majority of the cases.

Since the primary aim of viral load testing is the evaluation of treatment efficiency, the possible occurrence of false positive results in successfully treated patients is an important drawback. Increasing the cut off of the assay might be a possibility to avoid patients being falsely classified as treatment failures, but this would prevent the detection of real, early virological failure. False positive results occurred less frequently in the Generic HIV viral load assay. The Generic HIV viral load assay is an amplification-based assay and does not contain an internal decontamination step comparable to the UNG-decontamination in the Amplicor Monitor. Although amplification and detection is performed in a single, sealed plate, which reduces the risks for contamination, false positive results due to PCR contamination cannot be excluded, especially in laboratories where strict separation between sample processing area, pré-PCR area and amplification area is not obvious.

When developing assays for resource-limited countries, special attention should be paid to the high genotypic diversity of HIV in these regions. Subtyping was performed on a subset of the samples used in this study and revealed the presence of at least five subtypes and a number of unique recombinations. The subtype distribution found was similar to what was previously described for the same region (Neilson et al., 1999; Yang et al., 2004). No subtype-related differences were expected for the ExavirLoad assay, as the measurement of the RT activity is supposed to be independent of the HIV-subtype (Braun et al., 2003; Jennings et al., 2005; Malmsten et al., 2003, 2005; Seyoum et al., 2006; Sivapalasingam et al., 2005; Stevens et al., 2005). Nevertheless, infections with subtype D and C virus were associated with five and two false positive results, respectively. Due to a small sample size for subtype D, C, CRF16_AD, F and the recombinant forms, statistical analyses of single subtype specific differences was not possible. When grouping all non-A subtypes, no statistically difference between the performance of the ExavirLoad assay for subtype A and non-A was observed ($p = 0.699$). Additional studies are needed to evaluate the performance of the ExavirLoad assay for each of the subtypes individually.

For the Generic HIV viral load assay, no effect of HIV subtype on the performance of the test could be observed. This is in

Table 3
Results of viral load assays according to subtype

Study no.	Subtype	Amplicor Monitor		Generic HIV viral load		ExavirLoad	
		VL (c/ml)	VL (log c/ml)	VL (c/ml)	V (log c/ml)	VL (c/ml)	VL (log c/ml)
020	A	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
133	A	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
116	A	>100,000	>5.00	>100,000	>5.00	79,150	4.90
018	A	35,400	4.55	55,272	4.74	68,850	4.84
139	A	26,300	4.42	4,811	3.68	2,526	3.40
028	A	21,600	4.33	55,472	4.74	11,655	4.07
011	A	16,900	4.23	51,926	4.72	42,350	4.63
141	A	10,400	4.02	1,000	3.00	1,866	3.27
016	A	5,330	3.73	2,367	3.37	3,056	3.49
106	A	3,520	3.55	6,802	3.83	1,186	3.07
144	A	2,520	3.40	5,152	3.71	4,104	3.61
132	A	2,260	3.35	2,524	3.40	485	2.69
127	A	1,690	3.23	630	2.80	2,119	3.33
102	A	1,250	3.10	8,196	3.91	5,490	3.74
113	A	1,030	3.01	1,000	3.00	1,914	3.28
145	A	294	2.47	<300	<2.48	2,140	3.33
039	A	159	2.20	4,811	3.68	1,179	3,07
142	A	128	2.11	<300	<2.48	463	2.67
021	A	73	1.86	<300	<2.48	<400	<2.60
101	A	<50	<1.70	<300	<2.48	<400	<2.60
111	A	<50	<1.70	<300	<2.48	<400	<2.60
119	A	<50	<1.70	<300	<2.48	<400	<2.60
121	A	<50	<1.70	<300	<2.48	<400	<2.60
130	A	<50	<1.70	<300	<2.48	<400	<2.60
136	A	<50	<1.70	<300	<2.48	<400	<2.60
143	A	<50	<1.70	<300	<2.48	<400	<2.60
122	A	<50	<1.70	<300	<2.48	3,410	3.53
134	A	<50	<1.70	<300	<2.48	1,390	3.14
148	A	<50	<1.70	<300	<2.48	1,301	3.11
118	A	<50	<1.70	<300	<2.48	834	2.92
138	A	<50	<1.70	<300	<2.48	408	2.61
030	C	66,300	4.82	43,205	4.64	40,125	4.60
105	C	21,000	4.32	10,576	4.02	14,690	4.17
120	C	81	1.91	<300	<2.48	1,272	3.10
131	C	<50	<1.70	<300	<2.48	1,723	3.24
117	C	<50	<1.70	<300	<2.48	494	2.69
103	C	<50	<1.70	3,833	3.58	<400	<2.60
108	D	74	1.87	<300	<2.48	<400	<2.60
115	D	<50	<1.70	<300	<2.48	<400	<2.60
129	D	<50	<1.70	<300	<2.48	1,265	3.10
150	D	<50	<1.70	<300	<2.48	1,113	3.05
110	D	<50	<1.70	<300	<2.48	456	2.66
112	D	<50	<1.70	<300	<2.48	421	2.62
109	D	<50	<1.70	<300	<2.48	404	2.61
126	G	<50	<1.70	<300	<2.48	989	3.00
002	CRF16_AD	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
147	CRF16_AD	98,200	4.99	>100,000	>5.00	5,480	3.74
114	CRF16_AD	15,300	4.18	1,080	3.03	<400	<2.60
107	CRF16_AD	55	1.74	<300	<2.48	<400	<2.60
104	CRF16_AD	<50	<1.70	<300	<2.48	<400	<2.60
125	CRF16_AD	<50	<1.70	<300	<2.48	1,136	3.06
140	CRF16_AD	<50	<1.70	<300	<2.48	490	2.69
010	A +D	9,790	3.99	7,415	3.87	<400	<2.60
123	A + D	3,060	3.49	518	2.71	2,810	3.45
137	AE + D	<50	<1.70	<300	<2.48	<400	<2.60
128	A + C	<50	<1.70	<300	<2.48	892	2.95

VL = Viral load, c/ml = copies/ml.

agreement with other studies evaluating an in-house real-time PCR assay that targeted the same Long Terminal Repeat (LTR) region. The LTR region is one of the more conserved regions of the HIV-1 genome and therefore perfectly suitable for the development of assays with broad subtype specificity (Drosten et al., 2006; Rouet et al., 2007, 2005).

The strength of the study presented here is that the assays were evaluated in 'field conditions' and in an adequate number of both ART naïve and experienced patients. Only few other studies have addressed the use of the ExavirLoad or the Generic HIV viral load assay or a comparable in-house LTR based real-time PCR assay for the follow-up of adult patients on ART (Braun et al., 2003; Greengrass et al., 2005; Stevens et al., 2005). They concluded that both assays could be suitable to monitor treatment in resource-limited countries. Sample sizes were, however, very small and all these studies were conducted in well-equipped laboratories which might explain the better performance of these assays compared to the results presented here.

The observation of some false positive results in HIV-negative patients might impair the use of the Generic HIV viral load assay for diagnosis of perinatal HIV infection although an in-house LTR based real-time PCR assay has also shown to be highly sensitive and specific for early pediatric diagnosis (Rouet et al., 2005).

When evaluating viral load tests in resource-limited settings, one should not only take into account the performance of the test, but also recurrent costs, availability of trained laboratory staff, logistics for reagent delivery, accessibility of instrument servicing and availability of external quality control programs. The ExavirLoad assay has the advantage of a relatively cheap start-up cost (US$ 3000) and maintenance free equipment. However, the turn around time of the assay is long (2.5 days for 30 samples) and the test is very labor intensive. Even though the cost per test (US$ 30) is lower than the current commercially available assays, it remains relatively high. To our knowledge, there is no external quality control program available which includes the ExavirLoad assay. A possible advantage of the test is the option of using the left-over of the extracts in a kit for phenotypic resistance analysis (HIV Phenotype RT kit) (Shao et al., 2003; Tuaillon et al., 2004).

The turn around time of the Generic HIV viral load assay is low, allowing 48 samples to be run in one day. The cost per test is about US$ 20 but can safely be reduced to US$ 10 by reducing the recommended volume reagent as appeared from the results described, although a more thorough evaluation of this adaptation is needed. Purchasing and servicing of the PCR equipment, however, remains a high cost to overcome. An advantage might be that the same equipment can be used to perform other real-time PCR assays like assays for the detection of other sexually transmitted infections.

In conclusion, both the Generic HIV viral load assay and the ExaVirLoad assay could be suitable tools for viral load determination in resource-limited settings, yet both tests have their limitations and should not be implemented without a thorough on-site evaluation. Further research is needed to address the influence of different subtypes on the performance on the evaluated viral load assays, especially for ExavirLoad. More research is needed to optimize the sensitivity and specificity of low-cost viral load assays and to develop assays that are easy to handle and require only a minimum of laboratory infrastructure and training. Meanwhile manufacturers of commercial viral load assays should commit themselves to further simplify their assays, reduce the prices and provide the necessary support in infrastructure for their use in resource-limited settings.

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2.2. A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance

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A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance

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Abstract

Quantification of the viral burden and identification of drug resistant mutations are important laboratory tools in the management of HIV-1 infected patients. However, widespread use of assays for viral load determination and genotyping is still hampered by the high cost. Here, an in-house RT-PCR-sequencing assay for HIV-1 drug resistance monitoring with the potential to be used both as a qualitative assay to detect the virus in plasma and as a genotyping system is described. A total of 377 clinical samples, collected from 374 HIV-infected patients of diverse geographic origin, were tested. The nested RT-PCR for amplification of the protease reverse transcriptase gene was found positive for 350 (92.8%) and 346 (91.8%) of 377 samples, respectively. All amplification-failures were due to viral loads of below 500 copies/ml. However, low viral load does not exclude amplification since 80.2 and 76% of 121 samples with viral loads of less than 500 copies/ml were amplified successfully for protease and reverse transcriptase, respectively. The high sensitivity of the assay was independent of the HIV-subtype, with a broad range of different HIV-1 subtypes tested. In conclusion the RT-PCR-direct sequencing method is convenient for the sensitive detection and subsequent genotyping of plasma RNA from a broad range of different HIV-1 subtypes. The assay enables the accurate follow-up of patients under treatment at a significantly reduced cost compared to the currently available commercial assays for viral load assessment and genotyping.

Keywords: HIV-1; RT-PCR; Genotyping; Qualitative detection

1. Introduction

Highly active antiretroviral therapy (HAART), based mainly on combinations of reverse transcriptase (RT) and protease (PR) inhibitors is widely used to treat human immunodeficiency virus (HIV) infections. Whenever the virus is able to continue replication in the presence of these drugs, drug-resistant variants emerge, and the response to therapy is lost. Drug-resistant variants that evolve during treatment can be transmitted from one person to another and such spread of drug-resistant strains through the population compromises the future use of any antiretroviral drug (Hanna et al., 2003; Tang and Pillay, 2004). Continuous monitoring of therapeutic effects and of development of drug resistance in patients under therapy is therefore of utmost importance.

Infrastructure and financial resources to ensure follow-up of treatment, with regular measurements of the viral load and CD4 count and testing for drug resistance, are still lacking in most parts of the world. Both, viral load assays and genotyping systems for detection of drug resistance are commercially available, but their use in poorly resourced environments is hampered by the high cost.

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The aim of this study was to design and evaluate a RT-PCR-genotyping system for the sensitive amplification and sequencing of the protease (PR) and the reverse transcriptase (RT) gene from a broad variety of different HIV-1 subtypes. Although the main objective was to develop a low-cost alternative for the commercial genotyping systems, the idea was also that a very sensitive RT-PCR would allow combined screening for viral replication in plasma and immediate sequencing of drug resistance in the replicating virus. This might be the ideal combination for a fast and low-priced monitoring of patients under treatment.

2. Material and methods

2.1. Patients and samples

For this study, 377 samples from 374 HIV-1-infected patients were selected. Two hundred and forty five samples were from the patient cohort of the Aids Reference Center at Ghent University Hospital in Belgium. For 174 of these samples genotyping was requested by the physician as part of the routine screening for baseline resistance in therapy naïve patients ($n = 106$) or because of indications for therapy failure ($n = 68$). The remaining 71 samples were included because of viral loads below 500 copies/ml. In addition, 95 samples were obtained from treatment naïve patients visiting the Day Clinic of the Yaoundé Central Hospital, Cameroon, and 37 samples were from HIV-1 infected therapy-naïve women attending the Coast General Hospital in Mombasa, Kenya. The latter samples were collected in the non-intervention arm of a study performed during 1996–1999 examining the effect of vaginal lavage with diluted chlorhexidine on mother-to-child transmission (Gaillard et al., 2001). Twelve samples with a viral load of <50 copies/ml were also included.

Blood was collected in EDTA-treated tubes and the plasma was stored at −20 to −80 °C before use. Plasma viremia was quantified in the samples collected in Belgium and in Kenya using the Cobas Amplicor Cobas Ampliprep HIV-1 Monitor Assay, version 1.5 (Roche Diagnostics, Basel, Switzerland).

2.2. HIV RNA extraction

Viral RNA was extracted with the High Pure nucleic acid kit (Roche Molecular Biochemicals, Mannheim, Germany). Virus in the plasma was first concentrated by centrifugation of an aliquot of 500 μl plasma at 23,600 × *g* at 4 °C for 1 h. After removal of the plasma, the pellet was suspended in 200 μl phosphate buffered saline (PBS). Subsequent extraction was performed according to the manufacturer's instructions with a final elution of the RNA in 50 μl of the elution buffer. In each extraction, a pool of HIV positive plasma samples, diluted in negative plasma until a viral load of 1000 copies/ml is obtained, is included as a positive control. Plasma from a HIV negative blood donor is included as a negative control. The PCR result for the positive control has to be positive, otherwise all negative samples from that run must be repeated.

2.3. Amplification of HIV RNA by nested RT-PCR

An outer gag-pol RT-PCR product was obtained using the Titan One tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany) with a combination of four primers. The RT-PCR was performed using 10 μl of purified viral RNA in a 50 μl volume reagent mix containing the RT-PCR buffer, 100 mM dithiothreitol and 1 μl of the Titan enzyme mixture (AMV reverse transcriptase, Taq DNA polymerase and Pwo DNA polymerase) and further supplemented with 25 mM deoxynucleoside triphosphates (Amersham Biosciences, Buckinghamshire, UK), 40 U/μl of RNase inhibitor (Promega Biosciences, San Luis Obispo, CA) and 0.1 μM (each) of two forward primers (GAG2: 5′-GAGGAAGCTGCAGAATGGG-3′ and PR1: 5′-ATGATGCAGAGAGGCAATTT-3′) and the two reverse primers (RT137: 5′-TTCTGTATGTCATTGACAGTCCAGC-3′ and RT3303: 5′-TAAYTTYTGTATRTCATTGAC-3′). Primers were selected to have a broad specificity for different HIV-1 subtypes.

The RT reaction was carried out at 45 °C for 30 min and 94 °C for 2 min and immediately followed by the DNA amplification: 10 cycles at 94 °C for 30 s, 45 °C for 30 s and 68 °C for 1 min; 10 cycles at 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min; 15 cycles at 94 °C for 30 s, 55 °C for 30 s and 68 °C for 3 min and a final extension for 7 min at 68 °C.

A 2 μl volume of the outer RT-PCR products were used in two separate inner PCR reactions, one with nested protease primers (PR3: 5′-AGAGCCAACAGCCCCACCA-3′ and PR4: 5′-GGGCCATCCATTCCTGGCTT-3′) and one with nested reverse transcriptase primers. (RT1: 5′-CCAAAAGTTAAACAATGGCCATTGACAGA-3′ and RT4: 5′-AGTTCATAACCCATCCAAAG-3′). The second-round PCR was carried out using Taq polymerase (Applied Biosystems Incorporated, Foster City, CA) in a 50 μl reaction volume containing Taq buffer II with 5 U/μl of Taq Polymerase, 25 mM $MgCl_2$, 1.25 mM deoxynucleoside triphosphates (Amersham Biosciences, Buckinghamshire, UK) and 0.05 μM (each) of the specific forward and reverse primers. The second-round PCR included an initial denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s and a final extension for 7 min at 72 °C. The PR-primers amplify a 458 bp fragment spanning the whole PR gene. The RT-primers amplify a 646 bp fragment spanning the aminoacids 30 to 227 of the RT gene.

A GeneAmp PCR system 9700 thermal cycler (Applied Biosystems Incorporated, Foster City, CA) was used for all PCR reactions. The results were checked by electrophoresis of the nested PCR products on 1% agarose gels and visualized with ethidium bromide under UV light.

2.4. Sequencing

The RT-PCR products, which showed a clear band on the agarose gel, were purified by the QIAquick PCR Purification kit (QIAgen, Venlo, The Netherlands). The purification was performed according to manufacturer's instructions with a final elution in 50 μl of the elution buffer.

Purified PCR products were subjected to direct sequencing of both the sense and antisense strands using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems Incorporated, Foster City, CA). For each sample six separate sequencing reactions were performed using respectively the four inner PCR primers (PR3, PR4, RT1 and RT4) and two additional internal RT primers: RT2882: 5′-GGGNGAYGCATATTTTTCARTWCC-3′ and RT2949: 5′-CCTGGTGTYTCATTRTTTRYAC-3′. A 0.02 μM of each primer was used in a 20 μl volume mix containing 3 μl of the purified RT-PCR product, 3.5 μl 5× cycle sequencing dilution buffer, 1 μl cycle sequencing reaction mix and 10 μl ultrapure water. The sequencing PCR included 30 cycles of 95 °C for 30 s, 55 °C for 15 s and 60 °C for 4 min.

After running the sequencing reaction, non-incorporated dideoxynucleoside triphosphates were removed by ethanol–sodium acetate precipitation and the pellet was resuspended in 25 μl High Density Formamide (Applied Biosystems Incorporated, Foster City, CA) for denaturation and loading on the ABI 310 Genetic Analyzer (Applied Biosystems).

2.5. Validation, alignment and interpretation of sequencing results

All validations and subsequent manipulations of the sequencing results as well as the interpretations of the genotyping data were performed with software packages and genotyping algorithm programs that are freely available through the internet.

Electropherogram patterns were displayed and manually edited using ‘Chromas’ (http://www.technelysium.com.au/). Assembly and alignment was done with the BioEdit software package designed by Tom Hall (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Interpretation of the sequences according to resistance was performed using the Stanford HIV drug resistance database (http://www.hivdb.stanford.edu/).

2.6. Phylogenetic analysis

Phylogenetic analyses and neighbor-joining tree reconstructions were performed using programs from version 3.6 of the PHYLIP package (http://www.evolution.genetics.washington.edu/phylip.html), with a maximum-likelihood distance matrix and a transition to transversion ratio of 2.0. Tree diagrams were plotted with Treeview v1.4 (included in the PHYLIP package).

3. Results

3.1. Nucleic acid extraction and nested RT-PCR

RNA extraction and RT-PCR was attempted on a total of 377 samples of which 245 were collected in Belgium, 95 in Cameroon and 37 in Kenya. Viral load results were available for all samples from Belgium and Kenya, and varied respectively between 51 and >100,000 copies/ml (samples from Belgium) and between 17,000 and >100,000 copies/ml (samples from Kenya). Viral load determination was not performed systematically for the samples from Cameroon.

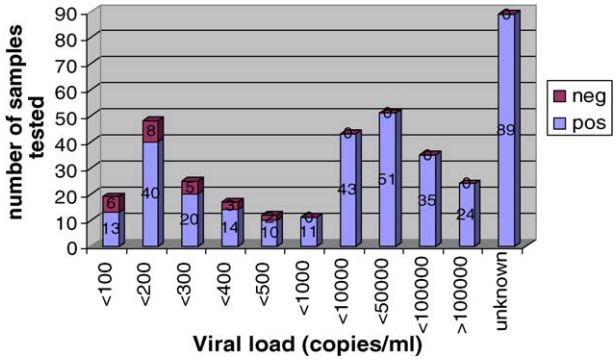

Fig. 1. Results of the RT-PCR for amplification of the protease gene. Neg, no amplification; Pos, successful amplification.

Successful amplification of the PR region was obtained in 350 samples, 223 from Belgium, 90 from Cameroon and 37 from Kenya, resulting in an overall success rate of 92.8%. For the RT region 346 samples were successfully amplified, 219 from Belgium, 90 from Cameroon and 37 from Kenya, resulting in an overall success rate of 91.8%. Of the 33 samples for which amplification failed, viral load results were available for 28 and appeared to be very low (<500 copies/ml). Viral loads were determined subsequently for the five additional samples for which amplification failed. Results were <50 copies/ml for three samples and respectively 204 and 424 copies/ml for the remaining two.

Additionally 12 samples with a viral load of <50 copies/ml were tested. All were found negative.

Although failure to amplify the PR and RT gene can occur in samples with a viral load of below 500 copies/ml, the majority (respectively 80 and 76%) of the 121 samples with a viral load below 500 copies/ml that were tested were successfully amplified for the PR and RT gene.

A summary of the RT-PCR amplification results for PR and RT is shown in Figs. 1 and 2.

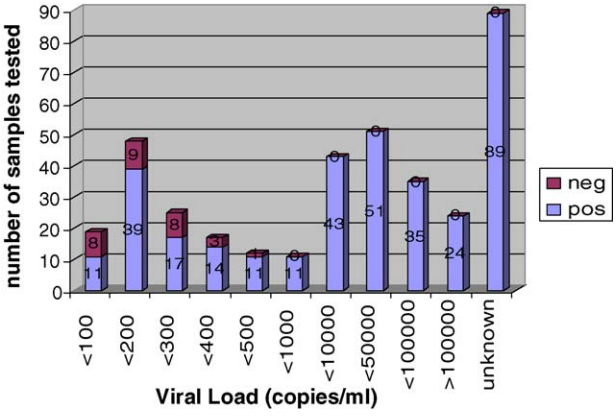

Fig. 2. Results of the RT-PCR for amplification of part of the reverse transcriptase gene. Neg, no amplification; Pos, successful amplification.

3.2. Genotyping

Sequencing of the 350 PR and 346 RT amplicons gave useful sequence data for all. A sequence of good quality for all six primers was obtained in 85.3% of the samples. Sequences that were partially single stranded due to failure of one or two sequencing primers were seen in 12.9 and 1.7% of the samples, respectively.

For 227 of the 346 patients with an available PR and RT sequence, including all 90 samples from Cameroon, all 37 samples from Kenya and 100 of the 219 samples from Belgium, genotyping was requested to assess the presence of baseline resistance. Only in 20 of these therapy naive patients, mutations at one of the positions associated with resistance in either the RT gene (codons 41, 44, 62, 65, 67, 69, 70, 74, 75, 77, 98, 100, 101, 103, 106, 108, 115, 116, 118, 151, 181, 184, 188, 190, 210, 215 and 219) or the PR gene (codons 30, 32, 33, 46, 48, 50, 53, 54, 73, 82, 84, 88 and 90) were detected (see Table 1). All mutations were single mutations and none of these isolated mutations on its own is expected to lead to a reduced response to any HAART regimen. In one patient however (BE03026) the observation of a T215D mutation in the RT gene indicated possible infection with an AZT resistant strain and a subsequent faster evolution towards resistance when an AZT containing regimen is eventually initiated.

Sixty-six patients, all sampled in Belgium, were screened because of therapy failure. Resistance inducing mutations were detected in 50. A mutation at position 215 of the RT gene was observed most frequently (24 patients) followed by the M to I or V substitution at position 184 (21 patients) (see Table 1). All mutations observed were in accordance with the expectations based on the current medication and/or the treatment history of the patient.

3.3. Detection of resistance in patients with low viral loads

Twenty-three of the 140 patients with a viral load below 1000 copies per ml were subjected to genotyping because of indications of therapy failure. A completely wild type virus was detected in six, including three patients (BE96031, BE96042 and BE92022) with samples collected during an occasional blip of the viral load, one patient (BE00041) in whom therapeutic drug monitoring revealed suboptimal lopinavir concentration and adaptation of the lopinavir dosage resulted in a subsequent drop of the viral load below the detection limits, and one patient (BE01011) suspected of bad therapy adherence.

Resistance mutations were detected in 18 patients. Twelve of them had a long history of treatment and treatment failure and the observed mutational patterns were comparable to the ones from previous blood drawings or were in accordance with the expectations based on the treatment history. In six patients (BE135812, BE99021, BE02061, BE89019, BE02028 and BE04025) genotyping was performed for a first regimen failure. The failing HAART regimens were AZT + 3TC + ABC (in BE02028), 3TC + ddI + TFV (in BE02061 and BE89019), d4T + 3TC + NFV (in BE135812), AZT + 3TC + NFV (in BE99021) and AZT + 3TC + LPV (in BE04025). In four patients (BE02028, BE99021, BE02061 and BE89019) the mutations were detected within 6 months after starting the treatment. The RT mutation M184I/V was detected in all six. Additional mutations observed were the RT substitutions K65R (in BE89019) and M41L (in BE135812) and the PR substitution L90M (in BE135812).

3.4. Performance of the RT-PCR for different HIV-1 subtypes

Fig. 3 shows a phylogenetic tree based on the RT sequences of all the patients for whom a sequence was obtained. Different subtypes reference sequences were included. Ten different subtypes were represented in the patient' cohort that was selected for the evaluation (A, B, C, D, CRF01_AE, F, G, CRF02_AG, H and J). Representatives of subtypes A, B, C, D, CRF01_AE, F, G, CRF02_AG, H, K and 06_CPX were also found amongst the 97 samples with viral loads below 500 copies/ml and positive RT-PCR results (55% subtype B, 12% subtype CRF02_AG, 8% subtype CRF02_AE, 6% subtype F, 5% subtype C, 4% subtype A, 4% subtype G, 2% subtype D and 1% subtype K, 1% subtype H and 1% 06_CPX).

3.5. Manipulations and costs of the procedure

Table 2 gives an overview of all the manipulation steps with indications concerning hands-on time and cost. Only the cost of reagents and disposables (current prices in Belgium) is taken into account in these calculations.

Necessary equipment to perform the test includes a cooled centrifuge able to run at high-speed, a thermal cycler, equipment to run agarose gels and an instrument for capillary electrophoresis or an automatic sequencer. All manipulations can be performed in a level 2 laboratory.

4. Discussion

With the improved availability of HAART in resource limited settings (James, 2001), the demand for accurate but affordable monitoring of patients under treatment is becoming a priority (Kent et al., 2003). Indeed, a better access to antiretroviral drugs without adequate control and monitoring, might result in widespread transmission of drug resistant virus. An epidemic of drug-resistant HIV strains in developing countries can only be prevented by proper measures that guarantee that patients are prescribed only highly effective drug combinations, promote a correct intake of the medication, support the uninterrupted supply of the drugs, ensure the availability of laboratory methods to monitor side-effects and the success or failure of the treatment, and provide clear guidelines for actions in case of therapeutic failure. Laboratory methods to monitor success or failure of therapy must include at least CD4 count and viral load determinations and preferentially detection of drug resistance. The access to commercial assays for viral load monitoring and for determination of drug resistance in Africa is still limited due to the high costs.

Table 1
List of all patients in whom one or more mutations at codon positions associated with resistance were detected

	Sample ID	Reverse transcriptase																																Protease									
		41M	44E	62A	65K	67D	69i	69T	70K	74L	75V	77F	98A	101K	103K	106V	108V	115Y	116F	118V	151Q	181Y	184M	188Y	190G	210L	215T	219K	30D	32V	33L	36i	46M	48G	50I	53F	54I	73G	82V/I	84I	88N	90L
Therapy naive - baseline resistance																																											
1	CAM003N																																	N									
2	CAM025B																														F												
3	CAM02DN																														F												
4	CAM018B													R																													
5	CAM021A		D																																								
5	CAM023A															I																												
7	CAM024B																			I																								
8	KE63281									A																					V												
9	KE63595							S								I																												
10	KE62161																																L											
11	BE03005																														V												
12	BE03012																														V												
13	BE03050																														F												
14	BE03007												S																														
15	BE03026												S														D																
16	BE03030															I																												
17	BE03042												S																														
18	BE03045							I																									x									
19	BE03046							S																																			
20	BE02002			V																											V												
Therapy failure																																										
1	BE97017																																								A		
2	BE95001					N		S	R																		F						V								
3	BE02028																						V																				
4	BE04025							S															V								F		I							A		M
5	BE135812	L																					V																				M
6	BE148811																						V																				
7	BE89011	L											G										V				Y																
8	BE93012	L									M	L	G										V			W	Y					L	V						A		M
9	BE94024	L			N			R		T		G								I		C	V		A	W	Y	D		F		L			L	V		A	V		
10	BE94025	L			N				I					N						I			V			W	F	W								V		T	V		
11	BE96033													N														M														M
12	BE97001													N									V																				
13	BE99021																						V																				
14	BE037610																						V						N												D	
15	BE96041					T	X	G						Q													F																
16	BE03025																										Y																
17	BE03053					N			R				G											L			I	Q															
18	BE00016													N																													
19	BE00043				R																					E																
20	BE00023															I				I																							
21	BE00048	L																								W	D																
22	BE02061																						I																				
23	BE116506																						V																				
24	BE045104	L																		I						W	Y																
25	BE076506			V						I	L								Y		M																						
26	BE89017	L																		I			V		S	W	Y									V	S	A			M
27	BE89019				R																		V																				
28	BE91007	L	D													I				I						W	Y																
29	BE91006													Q	N												Y																
30	BE90001					N			R																		Y																
31	BE92029					N			R														V				Q									V						M	
32	BE92022																			A																							
33	BE92021	L	D			N			R																	W	Y	E														
34	BE95010	L																					V			W	Y																
35	BE94001	L			N		D	R												I							F	Q				I				T		V		M	
36	BE95015	L			N			R					E													W	F			I		I			L	V		A			M	
37	BE95017																			I							Q															
38	BE95021					N			R																		F	Q														
39	BE96027							N	R										F				V														V	S		V		M	
40	BE95035	L								V				Q	N								V		A		Y	R										A			M	
41	BE97041					N		D	R																																	
42	BE97016					N			R											D							F	Q														
43	BE96044					N			R				G										V		A		Y	E														
44	BE96039																			I			V																				
45	BE95036																			I																							
46	BE00054														A																												
Therapy interruption																																										
47	BE98027	L																									C																
48	BE03010														N																												
49	BE03054														N																												
50	BE94005	L	D												N											W	C																

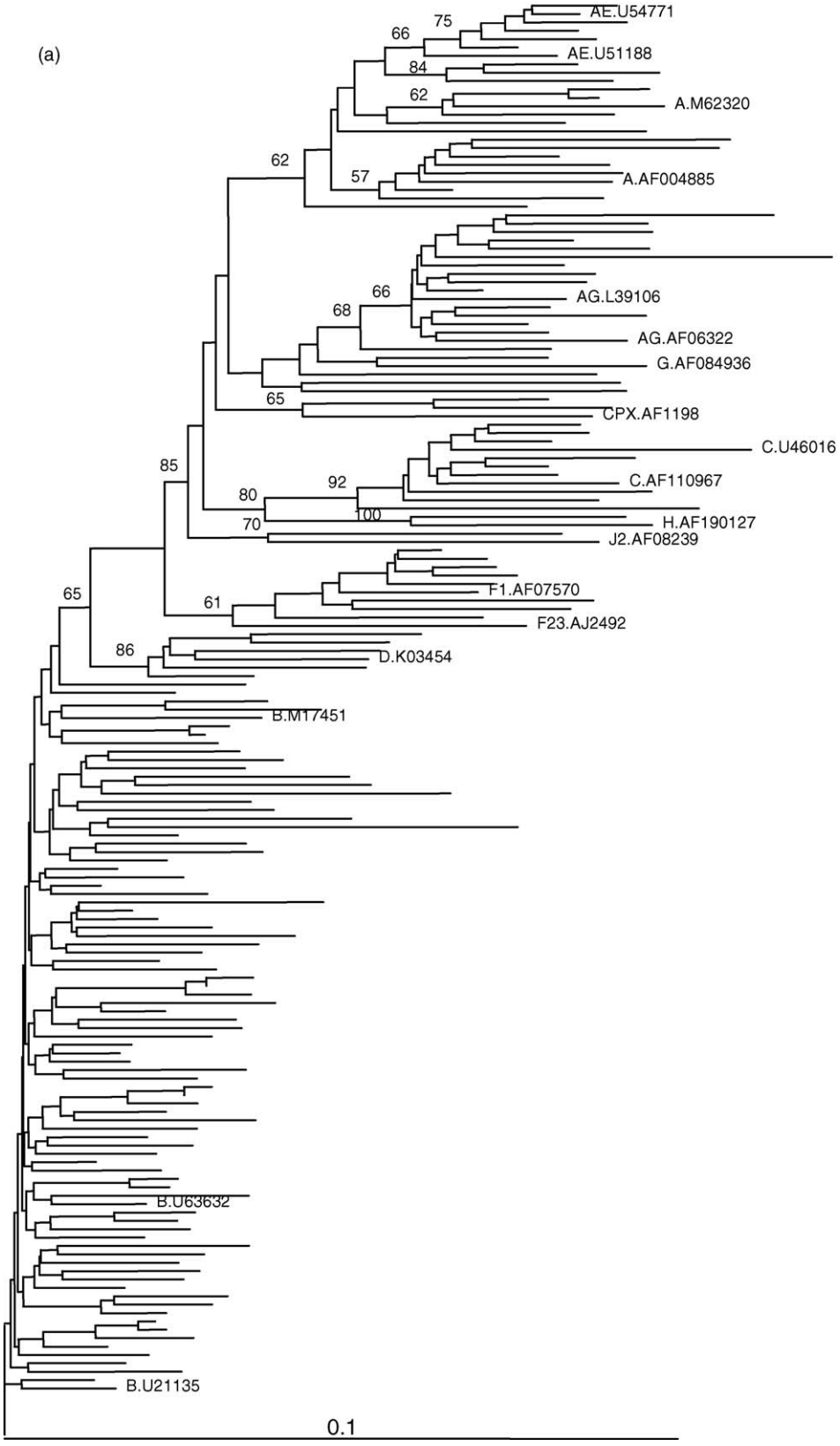

Fig. 3. Neighbor-joining tree based on the HIV-1 RT nucleotide sequences of the patients for whom successful amplification and sequencing was performed. (a) Sequences from samples collected in Europe (Belgium) and (b) sequences from samples collected in Africa (Cameroon and Kenya). The trees were constructed using the neighbor-joining method included in the Philip package. Numbers at the branch nodes indicate bootstrap values. For patients from whom 2 or 3 samples from different time points were included in the study, only one sequence was used for the construction of the phylogenetic tree. For sexual contacts known to be infected with the same virus (donor-recipient pairs) also only one sequence was included for phylogenetic analysis.

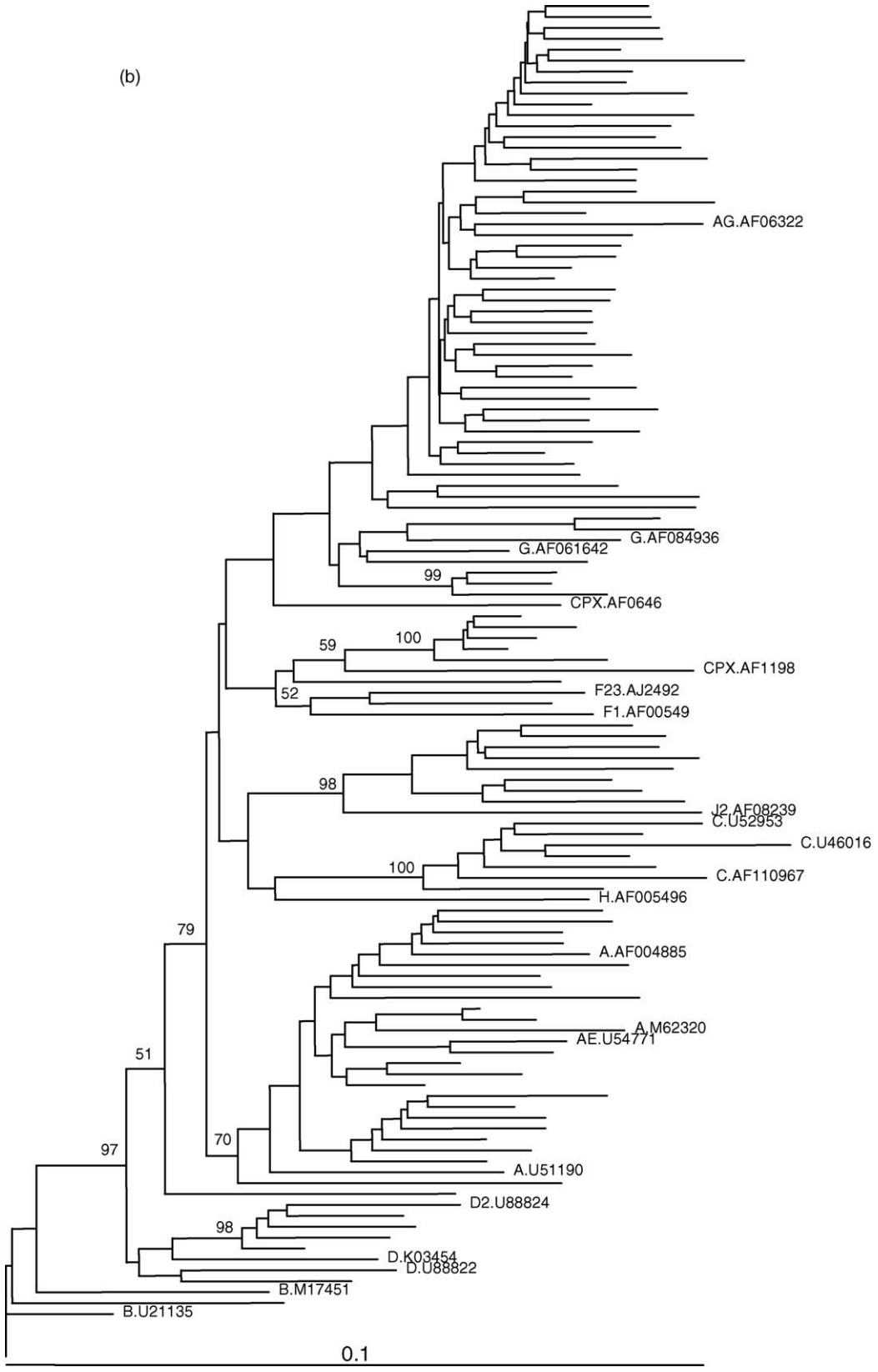

Fig. 3. (*Continued*).

Table 2
Hands-on time and costs of the different steps of the RT-PCR genotyping system

Product	Manipulations	Hands-on time	Cost per analysis (in Euro)
A. Detection of plasma HIV-1 RNA			
Plasma	Centrifugation	10 min	0.00
↓	RNA extraction	60 min	6.02
Purified RNA	RT-PCR	30 min	9.29
↓	Agarose gel electrophoresis	30 min	4.91
RT-PCR amplicon			
	Subtotal	2 h 10 min	19.50
B. Genotyping of HIV-1 RNA			
RT-PCR amplicon	Purification	30 min	2.69
↓	Sequencing reaction	20 min	22.79
Sequence amplicon	Purification	60 min	1.73
↓	Visualization	30 min	8.15
RT and PR sequence	Validation	20 min	0.00
	Interpretation	10 min	0.00
	Total	4 h 40 min	54.86

In this report an in-house genotyping method with high sensitivity for a large number of different HIV-1 subtypes is described. Although a well-equipped laboratory is still a necessity, this technique can be performed for a total reagent cost of 55 Euro, which is significantly lower than the commercially available systems. Moreover, the high sensitivity of RT-PCR step of the procedure, with a detection limit of below 500 copies/ml, permits its use as a qualitative screening test in patients under treatment. It also allows to discriminate between those with successful treatment (no detectable virus in the plasma) and those with therapy failure (detectable virus in the plasma), reducing the need for viral load determination. The costs for this qualitative RT-PCR alone are about 20 Euro with only a centrifuge and a thermal cycler needed. For the patients with detectable virus, genotyping of the amplified viral RNA for drug resistance can be initiated immediately for an additional cost of 35 Euro. The use of this method in the follow-up of patients under treatment will allow the fast detection of arising resistance at a more affordable price than the commercial tests.

It should be stressed that all commercial viral load and genotypic resistance assays currently available have been optimized for genetic subtype B strains while most of the infected patients worldwide are infected with non-B subtypes. And although at least the performance of two commercially available sequencing kits, the HIV Genotyping System kit and the TruGene™ HIV kit (Jagodzinski et al., 2003) can be considered as appropriate, problems with non-B strains are occasionally reported (Beddows et al., 2003; Fontaine et al., 2001; Mracna et al., 2001). Therefore the patient' cohort for evaluating the in-house method was specifically selected to embrace viral strains with a broad genetic diversity. Although subtype B is still the most prevalent in Belgium, it is responsible for only less than half of all the infections (Snoeck et al., 2002,2004). To further enlarge the non-subtype B group an important number of samples collected in Western Africa (Cameroon) and some samples collected in Eastern Africa (Kenya) were included. Several studies have shown the extensive subtype diversity of HIV-1 strains from Cameroon with an important representation of the recombinant form CRF02_AG and of the subtypes G, J and F (Fonjungo et al., 2002, Nkengasong et al., 1994). In Kenya, subtype A is the most prevalent, followed by subtypes D, C and G (Neilson et al., 1999).

The RT-PCR genotyping system proved to be highly successful for the amplification of African strains with positive results for 127 of the 132 African samples included. The five drop-outs had undetectable ($n = 3$) or very low ($n = 2$) viral loads in the Amplicor assay.

Commercial genotyping systems do not recommend the use of their assay for samples with viral loads of less than 1000 copies/ml. This recommendation is based on the fact that the success rate of the systems in low viral load samples is low and on the assumption that amplification of low viral load samples might lead to selection of non-representative strains. The latter assumption however was never supported by scientific data. Although the number of tested patients is limited, the results do indicate that genotyping as soon as therapy failure is suspected, despite extremely low viral loads, can be meaningful. In four patients resistance against one or two components of the HAART regimen was detected within 6 months after initiation, despite the fact that the viral load in these patients at the time of sampling was still extremely low (respectively 118, 169, 230 and 557 copies/ml). Not surprisingly, the observed resistance was against the components with the lowest threshold to resistance: 3TC (in all four) and tenofovir (in one).

The main objective of this report was to present an RT-PCR genotyping system for the monitoring of HIV-infected patients under treatment. The method described is able to amplify and sequence HIV-RNA from plasma samples from a random selection of patients encompassing different subtypes and an important number of patients with low viral loads. The method is therefore suitable for both detection of residual virus replication in the plasma and detection of drug resistance and can be performed at a significant lower price than the current commercial viral load and genotyping assays. Although the use of some expensive instrumentation is necessary, the equipment needed is versatile and can be used for other PCR and sequencing appli-

cations. A well equipped laboratory and sufficiently trained personnel remains however important. Time has come for the diagnostic industry and for the producers of laboratory equipment to follow the example of the pharmaceutical companies, which have supplied medication at affordable price for those countries where HIV is devastating to social and economical life. Now that therapy has become available, affordable follow-up of treatment and possible failure should be supplied urgently, such that therapies can be adopted and the rise of resistance among the population of viral strains can be kept at a safe level.

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2.3. Feasibility of detecting HIV-1 drug resistance in DNA extracted from whole blood or dry blood spots.

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Feasibility of Detecting Human Immunodeficiency Virus Type 1 Drug Resistance in DNA Extracted from Whole Blood or Dried Blood Spots▽

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Due to high cost, availability of human immunodeficiency virus type 1 (HIV-1) drug resistance testing in resource-poor settings is still limited. We therefore evaluated the usefulness of viral DNA extracted from either whole blood or dried blood spots (DBS). Samples were collected from 50 patients receiving therapy and 10 therapy-naïve patients. Amplification and sequencing of RNA and DNA was performed using an in-house assay. Protease (PR) and reverse transcriptase (RT) sequences of plasma viral RNA were obtained for 96.6% and 89.7%, respectively, of the 29 patients with a detectable viral load. For cellular viral DNA, useful PR and RT sequences were obtained for 96.6% and 93.1% of the whole-blood-cell samples and for 93.1% and 93.1% of the DBS samples, respectively. For the 31 patients with an undetectable viral load, PR and RT sequences were obtained for 67.7% and 61.3% of the whole-blood-cell DNA preparations and for 54.8% and 58.1% of the DBS DNA preparations, respectively. A good correlation between RNA and DNA sequences was found; most discordances were caused by the detection of mixed amino acids. Of the RT drug-resistant mutations, 13 (38.2%) were seen in RNA only, 6 (17.6%) in DNA only, and 15 (44.1%) in both. Repeated amplification and sequencing of DNA extracts revealed a lack of reproducibility for the detection of drug resistance mutations in a number of samples, indicating a possible founder effect. In conclusion, this study shows the feasibility of genotypic drug resistance testing on whole blood cells or DBS and its possible usefulness for HIV-1 subtyping or examining the overall distribution of drug resistance in a population. For individual patients, RNA sequencing was shown to be superior to DNA sequencing, especially for patients who experienced early treatment failure. The use of DNA extracted from whole blood or DBS for the detection of archived drug resistance mutations deserves further study.

Today, more than one million people in low- and middle-income countries have access to antiretroviral treatment (ART). Even though this is only 23% of the estimated 4.6 million human immunodeficiency virus type 1 (HIV-1)-infected individuals who are in need of highly active ART (HAART), it proves that the delivery of ART in resource-poor settings is feasible (41). Experiences from Europe and the United States, however, indicate that the emergence of HIV drug resistance remains an important obstacle to the long-term success of therapy. An adequate follow-up of patients on treatment, in order to identify cases in which therapy has failed, is essential to avoiding accumulation of drug resistance. Efforts to lower the prices for CD4 and viral load testing are being made, and alternative methods for measuring these parameters in resource-poor settings are being developed and evaluated (8, 12, 17). Unfortunately, genotypic resistance testing remains almost inaccessible due to its high cost, the need for specialized laboratories, and the logistical challenges, such as the requirements for a cold chain to transport samples from the field to reference laboratories. However, ART drug resistance testing in patients in whom treatment is failing remains very important, not only to guide a second line of treatment for individuals but also to allow the monitoring of the emergence and distribution of drug-resistant virus in the population.

In a previous study, we presented a sensitive method for combined detection of the presence of HIV-1 in plasma and subsequent sequencing of the protease (PR) and reverse transcriptase (RT) genes (36). Although this technique allows a significant reduction in the price of follow-up testing, the need for a proper infrastructure for collection and transportation of blood samples remains.

The aim of the current study was to evaluate the possibility of using cellular DNA instead of viral RNA for the detection of drug resistance mutations. As DNA is more stable than RNA, the use of DNA would eliminate the necessity for fast processing of the blood samples after collection and reduce the complexity of the manipulations. The fact that a reverse transcription step is no longer needed for the amplification of the genes of interest also reduces the overall cost of the procedure. Blood spots collected on filter paper have been used for DNA amplification purposes, but reports on the feasibility of performing genotypic analysis for HIV-1 drug resistance remain scarce (24, 33, 43). In resource-poor settings, dried blood spots (DBS) are the preferred method of sample collection in the

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field, as they do not require electricity or a cold chain. Moreover, once the samples are air dried on the paper, they can be handled as noninfectious and transported to the laboratory without the risk of infection transmission (19).

Whether the same resistance information can be obtained from DNA sequencing and RNA sequencing is still unclear. Results of published reports in this regard are very inconsistent (6, 10, 14, 21, 24, 34). The present study aims to investigate both the possibility of DNA sequencing from whole blood cells or from DBS and the usefulness of DNA sequencing for determination of drug resistance in patients from a resource-poor setting with a high level of HIV-1 subtype diversity.

MATERIALS AND METHODS

Patients and samples. In May 2006, a total of 50 patients on HAART and 10 ART-naïve patients were randomly selected from the HIV Comprehensive Care Centre at Coast Province General Hospital in Mombasa, Kenya. The patients were seen during regular follow-up visits, and written informed consent for participation in the study was obtained. The study was approved by the ethics review committees of the University of Nairobi and the Ghent University. The mean age of the patients was 36.9 years (standard deviation, ±9.2 cells/mm^3; range, 21 to 65 years), 38 (63%) of the patients were women, and 66% of the patients were in WHO clinical stage 3 or 4 (42). The mean CD4 count was 322 cells/mm^3 (standard deviation, ±166 cells/mm^3; range, 41 to 757 cells/mm^3). Patients on HAART were treated with the current regimen for a median of 12 months (ranging from 1 week to 33 months). The current regimen of two nucleoside RT inhibitors (NRTIs) (stavudine [d4T]/zidovudine [AZT] and lamivudine [3TC]), and one nonnucleoside RT inhibitor (NNRTI) (nevirapine [NVP] or efavirenz [EFV]), was the first-line regimen for 48 patients. Two patients received a second-line protease inhibitor (PI; lopinavir/ritonavir [LPV/r])-based regimen.

Ten milliliters of EDTA blood was collected, and 50 μl of whole blood was immediately spotted four times on filter paper (Schleicher and Schuell 903). Filter papers were air dried overnight and stored at −20°C until processing. CD4 cell counts were performed (FACScount; Becton Dickinson Immunocytometry, Oxford, United Kingdom), and the remainder of the EDTA blood was centrifuged to collect the plasma and the buffy coat cells. Plasma and whole blood cells were stored at −80°C and −20°C, respectively. All samples were shipped to the AIDS Reference Laboratory at the University Hospital in Ghent, Belgium.

RNA viral load testing. HIV RNA quantification was performed using the ultrasensitive Cobas Amplicor HIV-1 Monitor test, version 1.5 (Roche Molecular Systems, Branchburg, NJ), with a detection limit of 50 copies/ml.

DNA extraction. DNA was extracted from the blood cells by use of the QIAamp DNA blood mini kit (QIAGEN, Venlo, The Netherlands), following the instructions of the manufacturer, and eluted in 50 μl elution buffer.

The procedure to extract DNA from the DBS was adopted from that of Fisher et al. (15). Briefly, one whole DBS was cut into small pieces and transferred to a 2-ml tube. One milliliter of phosphate-buffered saline solution (Cambrex Bio Science, Verviers, Belgium) with 0.1% Tween 20 (Sigma-Aldrich, Bornem, Belgium) was added. The mixture was subjected to a vortex briefly, and after 10 min of incubation at room temperature, the supernatant was removed. This wash step was repeated twice. After the final wash step, 200 μl of 5% Chelex-100 resin solution (Bio-Rad, Nazareth, Belgium) was added and the sample was incubated for 30 min at 56°C and another 30 min at 95°C. The supernatant was then transferred to a clean tube and stored at −20°C until processing.

Amplification of RNA and DNA extracts. Amplification of HIV RNA was performed by nested RT-PCR as previously described (36). DNA extracts from the blood cells and the DBS were amplified by nested PCR using the same primer sets as the ones used for RNA amplification. The first round of amplification was carried out using 5 U/μl of *Taq* polymerase (Applied Biosystems Inc., Foster City, CA) in a 25-μl reaction volume containing buffer II (Applied Biosystems) with 25 mM $MgCl_2$, 1.25 mM deoxynucleoside triphosphates (Amersham Biosciences, Buckinghamshire, United Kingdom), 0.1% bovine serum albumin (Roche Diagnostics, GmbH Mannheim, Germany), and 0.05 μM each of two forward primers (GAG2 [5′-GAGGAAGCTGCAGAATGGG-3′] and PR1 [5′-ATGATGCAGAGAGGCAATTT-3′]) and two reverse primers (RT137 [5′-TTCTGTATGTCATTGACAGTCCAGC-3′] and RT3303 [5′-TAAYTTYTGT ATRTCATTGAC-3′]). Primers were selected to have a broad specificity for different HIV-1 subtypes.

Cycling conditions were 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min and a final extension for 7 min at 72°C. Two-microliter volumes of the outer RT-PCR products were used in two separate inner PCRs, one with nested PR primers (PR3 [5′-AGAGCCAACAGCC CCACCA-3′] and PR4 [5′-GGGCCATCCATTCCTGGCTT-3′]) and one with nested RT primers (RT1 [5′-CCAAAAGTTAAACAATGGCCATTGACAGA-3′] and RT4 [5′-AGTTCATAACCCATCCAAAG-3′]). The second-round PCR was carried out using *Taq* polymerase (Applied Biosystems Inc., Foster City, CA) in a 50-μl reaction volume containing *Taq* buffer II with 5 U/μl of *Taq* polymerase, 25 mM $MgCl_2$, 1.25 mM deoxynucleoside triphosphates (Amersham Biosciences, Buckinghamshire, United Kingdom), and 0.05 μM each of the specific forward and reverse primers. The second-round PCR included an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s and a final extension for 7 min at 72°C.

In each PCR run, a sample containing 10 copies of HIV-1 DNA (DNA equivalent of 10 8E5 cells) was included as a positive control. Ultrapure water was used as a negative control. Results were accepted only if the results of both controls were correct.

The PR primers amplify a 458-bp fragment spanning the whole PR gene. The RT primers amplify a 646-bp fragment spanning nucleotides corresponding to amino acids 27 to 227 of the RT gene. The amplification products were visualized after electrophoresis in a 1% agarose gel with ethidium bromide.

Genotyping of PR and RT genes. Genotyping was performed using a homemade sequencing assay as described earlier (36). Direct sequencing of both sense and antisense strands was done with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Foster City, CA). Sequencing reaction products were analyzed on an ABI310 or ABI3130XL genetic analyzer (Applied Biosystems Inc.). All validations and subsequent manipulations of the sequencing results, as well as the interpretations of the genotyping data and the subtyping, were performed using the Smartgene HIV software packages (Integrated Database Network System; Smartgene, Zug, Switzerland). The selection of drug resistance mutations was based on the Stanford algorithm (http://hivdb.stanford.edu).

Phylogenetic analyses. Phylogenetic analyses were performed using version 3.6 of the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html) with a maximum-likelihood distance matrix and a transition-to-transversion ratio of 2.0. Tree diagrams were plotted with Treeview, version 1.4 (included in the PHYLIP package). Nucleotide differences were calculated using the Emboss infoalign software (http://embossgui.sourceforge.net/demo/infoalign.html).

Statistical analysis. All statistical analyses were performed using SPSS 15.0 (SPSS, IL). Pearson's chi-square test was used to detect possible statistically significant differences between two groups of samples.

RESULTS

Viral load determination and RNA sequencing. A detectable viral load (>50 copies/ml) was found in all 10 treatment-naive patients (mean, 35,555 copies/ml; range, 73 to >100,000 copies/ml) and in 19 of the 50 treated patients (mean, 20,377 copies/ml; range, 55 to >100,000 copies/ml).

Sequencing of the viral RNA was attempted on all 29 of these samples and was successful for PR in 28 (96.6%) and for RT in 26 (89.7%). The three patients for whom one or both failed had very low viral loads (73, 74, and 81 copies/ml, respectively). The viral load was undetectable in 31 patients, all treated.

Efficiency of DNA sequencing on whole blood cells and DBS. Sequencing of the PR and RT genes from whole-blood-cell DNA was successful for 28 (96.6%) and 27 (93.1%), of the 29 blood-cell samples collected from patients with a detectable viral load, respectively, and for 21 (67.7%) and 19 (61.3%) of the 31 samples from patients with an undetectable viral load, respectively. For the DBS, the success rate was slightly lower, with 27 (93.1%) successful sequencing reactions for both PR and RT from the patients with a detectable viral load and 17 (54.8%) PR and 18 (58.1%) RT sequences from the patients with undetectable viral load. No statistically significant differ-

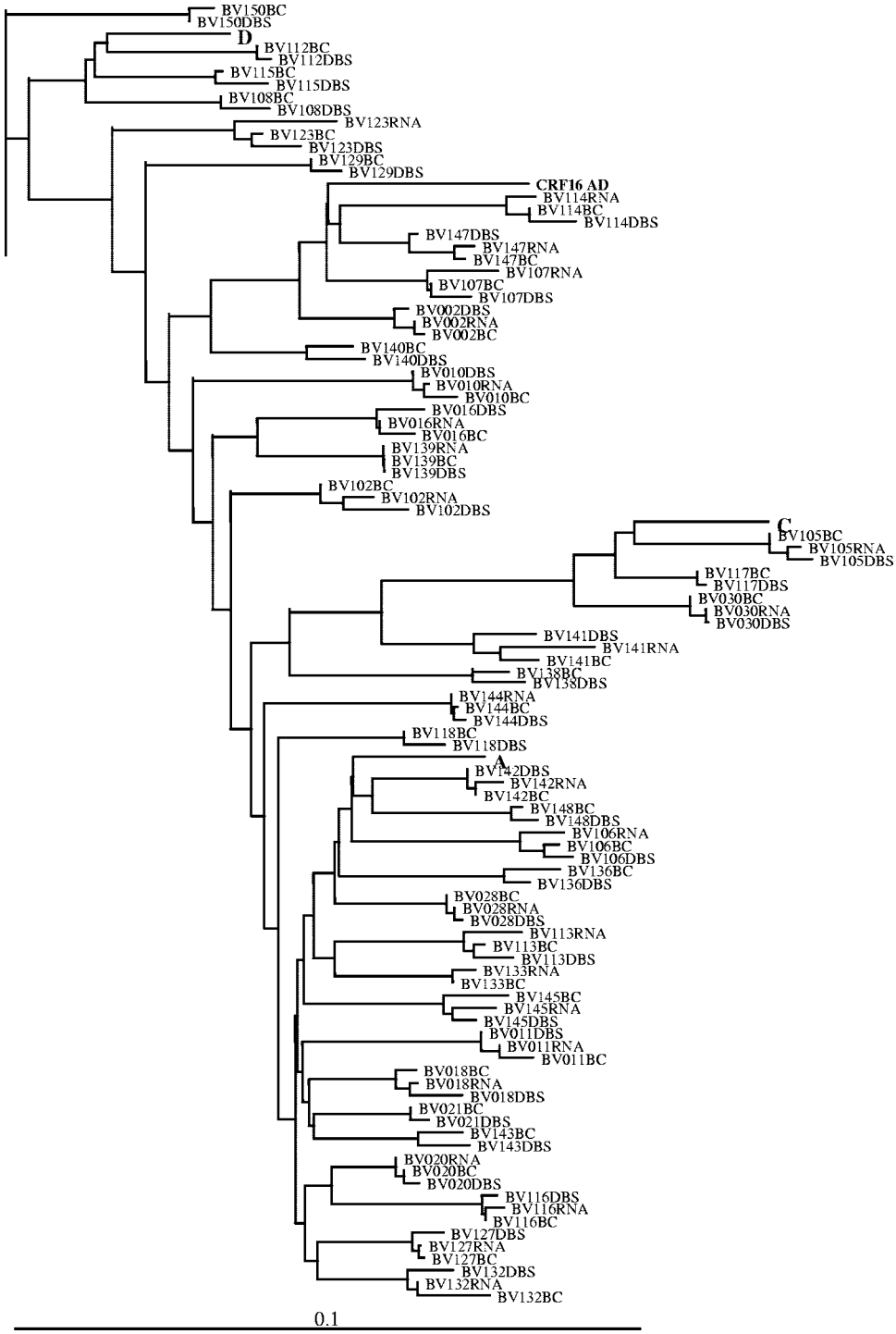

FIG. 1. A phylogenetic tree was constructed based on 100 PR and RT gene sequences from 38 patients. Reference strains for subtypes A, C, D, and CRF16_AD were included in the phylogenetic analysis. The tree was rooted with a subtype B strain. BC, blood cells.

ence between the efficiency of genotyping on whole blood DNA and the efficiency of genotyping on DBS DNA was found (P values of 0.2743 for PR and 0.8311 for RT [Pearson's χ^2 test]).

Subtype distribution. For the 56 patients from whom PR and RT sequences were available, from DNA, RNA, or both, subtyping of the HIV-1 strains was performed by similarity search using the Smartgene subtyping tool. Results showed that the majority of patients was infected with a subtype A virus ($n = 31$); in order of prevalence, subtype A was followed by subtypes D ($n = 7$), CRF16_AD ($n = 7$), C ($n = 6$), and G ($n = 1$). Discordances between the subtype determined from

the PR sequence and the subtype determined from the RT sequence were seen for four patients (PR/RT subtype combinations D/C, A/C, CRF_AE/C, and C/A). The results of the subtype determination were not influenced by the sample type.

Comparison of the nucleotide sequences from whole-blood DNA, DBS DNA, and plasma RNA. A phylogenetic tree was constructed using 100 PR and RT gene sequences from 38 patients (Fig. 1). The tree revealed a close clustering of the DNA and RNA sequences from the same patients in all cases. The mean nucleotide difference, expressed as the percentage of the total nucleotide length (857 bp), between paired DNA sequences of blood cells and DBS was 2.10% (range, 0.35% to 4.00%). The mean nucleotide difference between the sequences obtained from plasma RNA and blood-cell DNA and the mean nucleotide difference between the sequences obtained from plasma RNA and DBS DNA were 1.82% (range, 0.35% to 3.73%) and 1.90% (range, 0.58% to 3.50%), respectively. Mixtures of nucleotides (International Union of Pure and Applied Chemistry nomenclature) were considered differences. For comparison, the observed mean nucleotide differences between duplicate sequencing reactions on RNA and DNA were 1.45% (range, 0% to 3.37%) and 1.61% (range, 0% to 3.61%), respectively.

Drug resistance mutations in RNA and DNA. Selection of drug resistance mutations was based on the recent update of the International AIDS Society-USA list (20). Only two patients showed primary mutations in the PR gene (corresponding to L33F and D30N).

The mutation resulting in L33F was detected consistently in all three samples from the same treatment-naïve patient (BV021). The mutation resulting in D30N was detected as part of a mixed population in one of the DNA sequences of patient BV011 (Table 1).

Secondary PR mutations were observed in samples from all patients. Most of these mutations (74 of the 83) were consistently detected in the RNA and in both of the DNA sequences, eight mutations were detected in DNA only, and one mutation was detected in RNA only.

No LPV-related PI resistance-associated mutations were observed in the two patients on a PI-based regimen (BV114 and BV120) (Table 1).

No NRTI or NNRTI resistance-associated mutations were observed in samples from the 10 treatment-naïve patients. For the 43 of 50 treated patients for whom at least one RT sequence (RNA and/or DNA) was available, NRTI or NNRTI resistance-associated mutations were detected in 13 samples (Table 2). A total of 34 nucleotide substitutions were seen; 10 of these were detected consistently in the RNA and in both DNA sequences, 5 were detected in the RNA and in one of the DNA sequences, 3 were detected only in the DNA from blood cells, and 2 were detected only in the DBS DNA (Table 2).

Thirteen mutations were found exclusively in the RNA. Six mutations were detected in one or both of the DNA sequences but not in the RNA. A mutation resulting in a replacement with residue 103N was seen in the DNA sequence of two patients on an NNRTI-based regimen but with an undetectable viral load.

The number of resistance mutations detected in the DNA samples of five treated patients with a viral load below 5,000 copies/ml ($n = 4$) was clearly lower than the number of resistance mutations detected in the DNA samples of six treated patients with a viral load of more than 5,000 copies/ml ($n = 17$), but the difference did not reach statistical significance (Pearson's chi-square $P = 0.11$). The numbers of resistance mutations in the RNA in both groups, on the other hand, were comparable, with 12 and 16 mutations found in the RNA from patients with a viral load below 5,000 copies/ml and from patients with a viral load above 5,000 copies/ml (Pearson's chi-square $P = 0.83$), respectively.

Reproducibility of the DNA sequencing. Replicate amplification and sequencing reactions were performed on the DNA extracts of all individuals listed in Table 2, with the exception of one ART-naïve patient. The results showed the inconsistent detection of some mutations (Table 3). Five resistance mutations that originally were not detected in the DNA but were present in the RNA sample were picked up in at least one of the replicate DNA-sequencing reactions. However, despite replicate analysis, several important resistance mutations remained undetectable even after triplicate sequencing of the DNA: mutations resulting in the presence of 103N and 184V in patient BV141, mutations resulting in the presence of 103N and 184V in patient BV123, mutations resulting in the presence of 67N and 184V in patient BV113, and a mutation resulting in the presence of 65R in patient BV106.

Replicate sequencing confirmed the presence of the mutation resulting in the presence of 103N in the two patients with an undetectable viral load while on an NNRTI-based HAART regimen. The mutation resulting in the presence of 103N was consistently detected in all replicates of patient BV117 and in half of the replicates from patient BV112 (Table 3).

Reproducibility of the RNA-sequencing reactions. To evaluate the influence of sampling on RNA sequencing, replicate reactions were performed on four RNA samples (from patients BV113, BV123, BV132, and BV141). Results were much more consistent than those for DNA. In samples from patients BV123 and BV141, the same resistance mutations were detected in all reactions. For patient BV113, the mutation resulting in a D67N substitution was detected in only two of the four replicates. For patient BV132, the mutation resulting in a K103N substitution was detected in only two of the five replicates (results not shown).

DISCUSSION

In the last 25 years, DBS have been used for various purposes, including detection of HIV antibodies (2, 5, 18, 23, 31), detection of HIV antigen (22, 32), early diagnosis of perinatally infected infants (4, 15, 28, 30, 35), quantification of $CD4^+$ T cells (26), and quantification of viral plasma RNA (1, 3, 7, 9, 16, 25, 29). Few studies examined the possibilities of performing drug resistance testing on dried blood, plasma, or serum on filter paper (24, 33, 43). Because of the ease to collect, store, and transport DBS, use of DBS is the ideal method for blood sampling in resource-poor rural settings. This, combined with the possibility of directly amplifying the genes of interest, without the need for a reverse transcription step, therebyreducing both complexity and cost of genotyping procedures (price reduction of 12 U.S. dollars/test), is an important advantage in view of efforts to increase the accessibility of resistance testing in resource-poor regions.

TABLE 1. Overview of all patients with mutations at resistance-related positions in the PR gene

Patient	ART (length of treatment)	VL (copies/ml)[a]	CD4 (cells/mm^3)	Sample[b]	Residue encoded in place of indicated amino acid											
					10L	13I	16G	20K	30D	33L	36M	46M	63L	71A	73G	77V
BV021	None	73	474	DBS		V	E			F	I/T					I
				BC		V	E/G			F	T					I/V
				RNA		V	E			F	T					I
BV016	None	5,330	233	DBS		V	E				I					
				BC		V	E				I					
				RNA		V	E				I					
BV010	None	9,790	702	DBS		V/L					I					
				BC		V					I					
				RNA		V					I					
BV011	None	16,900	390	DBS	V	V					I			T		
				BC	V	V			N/D		I					
				RNA	V	V					I					
BV028	None	21,600	473	DBS		I/V					I					
				BC		V					I					
				RNA		V					I					
BV018	None	35,400	246	DBS	I	V					I	I/M			S	
				BC	I	V					I					
				RNA	I	V					I					
BV030	None	66,300	109	DBS							I					
				BC							I					
				RNA							I					
BV002	None	>100,000	136	DBS		V	E				I					I
				BC		V	E				I					I
				RNA		V	E				I					I
BV020	None	>100,000	165	DBS	I/V	V					I		P/L			
				BC	I/V	V					I		L/V			
				RNA	I/V	V					I		L/V			
BV107	d4T + 3TC + EFV (6 mo)	55	338	DBS		V	E	R			I			T		
				BC		V	E	R			I			T		
				RNA		V	E	R			I			T		
BV120	AZT + 3TC + NVP (41 mo), d4T + 3TC + LPV/r (9 mo)	81	226	DBS							I		P			
				BC							I		P			
				RNA							I		P			
BV142	AZT + 3TC + NVP (2 mo)	128	236	DBS		V		R			I		P/L			
				BC		V		R			I					
				RNA		V		R			I					
BV145	d4T + 3TC + EFV (33 mo)	294	206	DBS		V					I					
				BC		V					I					
				RNA		V					I					
BV113	d4T + 3TC + EFV (32 mo)	1,030	467	DBS	V	V	E				I					
				BC	I	V	E				I					
				RNA	V	V	E				I					
BV102	d4T + 3TC + NVP (1 wk)	1,250	111	DBS	V	V					I					
				BC	V/L	V					I					
				RNA	V	V					I					
BV127	d4T + 3TC + EFV (3 mo)	1,690	314	DBS		V					I					
				BC		V					I					
				RNA		V					I					
BV132	d4T + 3TC + EFV (2 wk)	2,260	41	DBS				R			I					
				BC				R			I					
				RNA		I/V		R			I					
BV144	d4T + 3TC + NVP (2 mo)	2,520	182	DBS							I					
				BC							I					
				RNA							I					
BV123	d4T + 3TC + NVP (8 mo)	3,060	321	DBS		V					I		P			
				BC		V		R			I					
				RNA		V		R			I					
BV106	d4T + 3TC + NVP (23 mo)	3,520	300	DBS		V					I		P			
				BC		V					I		P			
				RNA		V					I		P			
BV141	d4T + 3TC + NVP (13 mo)	10,400	388	DBS							I					
				BC							I					
				RNA							I					
BV114	d4T + 3TC + EFV (34 mo), AZT + 3TC + LPV/r (1 mo)	15,300	217	DBS		V	E	R			I					
				BC		V	E	R			I					
				RNA		V	E	R			I					
BV105	AZT + 3TC + EFV (9 mo)	21,000	276	DBS							I		P			
				BC							I/M		P			
				RNA							I		P			
BV139	d4T + 3TC + EFV (1 wk)	26,300	556	DBS		V					I					
				BC		V					I					
				RNA		V					I					
BV147	d4T + 3TC+EFV (13 mo)	98,200	218	DBS		V	E				I					
				BC	I	V	E				I					
				RNA	I/L	V	E				I					
BV116	d4T + 3TC + NVP (22 mo)	>100,000	353	DBS	V	V		R			I					
				BC	V	V		R			I					
				RNA	V	V		R			T					
BV133	d4T + 3TC+NVP (8 mo)	>100,000	165	DBS	ND[c]	ND	ND				ND					
				BC	I/V	V	E				I					
				RNA	V	V	E				I					

[a] VL, viral load.
[b] RNA, plasma RNA; BC, blood-cell DNA; DBS, dried blood spot DNA.
[c] NA, not applicable.

TABLE 2. Overview of all patients with mutations at resistance-related positions in the RT gene

Patient	ART (length of treatment)	VL (copies/ml)[a]	CD4 (cells/mm^3)	Sample[b]	Residue encoded in place of indicated amino acid														
					65K	67D	69T	70K	75V	103K	108V	151Q	181Y	184M	188Y	190G	215T	219K	225P
BV145	d4T + 3TC + EFV (33 mo)	294	206	DBS						N									
				BC						N									
				RNA						N	I			V					
BV113	d4T + 3TC + EFV (32 mo)	1,030	467	DBS															
				BC															
				RNA		N								V					
BV132	d4T + 3TC + EFV (2 wk)	2,260	41	DBS						N									
				BC	R								C						
				RNA			N/T			K/N			C/Y						
BV123	d4T + 3TC + NVP (8 mo)	3,060	321	DBS															
				BC															
				RNA						N				V					
BV106	d4T + 3TC + NVP (23 mo)	3,520	300	DBS															
				BC															
				RNA	R/K									V					
BV141	d4T + 3TC + NVP (13 mo)	10,400	388	DBS															
				BC															
				RNA						N				V					
BV114	d4T + 3TC + EFV (34 mo), AZT + 3TC + LPV/r (1 mo)	15,300	217	DBS															
				BC						K/N									
				RNA						N									H
BV105	AZT + 3TC + EFV (9 mo)	21,000	276	DBS						N				V					
				BC						K/N				M/V					
				RNA						N				V					
BV147	d4T + 3TC + EFV (13 mo)	98,200	218	DBS					I/V	K/N	I/V							K/E	
				BC					I/V	N				M/V					H/P
				RNA						N				V			F		
BV116	d4T + 3TC + NVP (22 mo)	>100,000	353	DBS				R		N				V					
				BC						N				V					
				RNA						N				V					
BV133	d4T + 3TC + NVP (8 mo)	>100,000	165	DBS			N					K/M/Q/L		V	L	A			
				BC			N/T							M/V	L	A			
				RNA			N/T					M		V	L	A			
BV112	AZT + 3TC + NVP (1 mo)	<50	242	DBS															
				BC						K/N									
BV117	d4T + 3TC + NVP (21 mo)	<50	328	DBS						K/N									
				BC						K/N									

[a] VL, viral load.
[b] RNA, plasma RNA; BC, blood-cell DNA; DBS, dried blood spot DNA.

TABLE 3. Results of replicate amplification and sequencing reactions performed on DNA extracts

Patient	ART (length of treatment)	VL (copies/ml)[a]	CD4 (cells/mm^3)	Sample[b]	Residue encoded in place of indicated amino acid															
					65K	67D	69T	70K	75V	101K	103K	108V	151Q	181Y	184M	188Y	190G	215T	219K	225P
BV145	d4T + 3TC + EFV (33 mo)	294	206	DBS1							N									
				DBS2							N/K									
				DBS3							N/K				M/V					
				BC1							N									
				BC2							N				V					
				BC3							N									
				RNA							N	I			V					
BV113	d4T + 3TC + EFV (32 mo)	1,030	467	DBS1																
				DBS2																
				DBS3																
				BC1																
				BC2																
				BC3																
				RNA		N									V					
BV132	d4T + 3TC + EFV (2 wk)	2,260	41	DBS1							N									
				DBS2			N							C						
				DBS3	R									C						
				BC1	R									C						
				BC2	R									C						
				BC3			N							C						
				RNA			N/T				K/N			C/Y						
BV123	d4T + 3TC + NVP (8 mo)	3,060	321	DBS1																
				DBS2																
				DBS3																
				BC1																
				BC2							K/R									
				BC3																
				RNA							N				V					
BV106	d4T + 3TC + NVP (23 mo)	3,520	300	DBS1																
				DBS2											V					
				DBS3		H/D														
				BC1																
				BC2																
				BC3																
				RNA	R/K										V					
BV141	d4T + 3TC + NVP (13 mo)	10,400	388	DBS1																
				DBS2																
				DBS3																
				BC1																
				BC2																
				BC3																
				RNA							N				V					
BV114	d4T + 3TC + EFV (34 mo), AZT + 3TC + LPV/r (1 mo)	15,300	217	DBS1																
				DBS2							N	I/V								
				DBS3							N						A/G			
				BC1							K/N									
				BC2							K/N							T/A		
				BC3							N						A			H
				RNA							N									H
BV105	AZT + 3TC + EFV (9 mo)	21,000	276	DBS1							N				V					
				DBS2							N	I			V					
				DBS3																
				BC1							N/K				V/M					
				BC2							N	I/V			V					
				BC3							N	I/V			V/M					
				RNA							N				V					
BV147	d4T + 3TC + EFV (13 mo)	98,200	218	DBS1					I/V		K/N	I/V							K/E	
				DBS2							N									
				DBS3																
				BC1					I/V		N				M/V					H/P
				BC2							N				V			F		H
				BC3			D				N									H
				RNA							N				V			F		
BV116	d4T + 3TC + NVP (22 mo)	>100,000	353	DBS1				R			N				V					
				DBS2					I/V		N	I/V			V					
				DBS3							N				V					
				BC1							N				V					
				BC2							N				V					
				BC3							N				V					
				RNA							N				V					
BV133	d4T + 3TC + NVP (8 mo)	>100,000	165	DBS1			N						M/Q/K/L		V	L	A			
				DBS2						K/E					V	L	A			
				DBS3																
				BC1			N/T								M/V	L	A			
				BC2											V	L/F	A			
				BC3			N						M		V	L	A			
				RNA			N/T						M		V	L	A			

Continued on facing page

TABLE 3—*Continued*

Patient	ART (length of treatment)	VL (copies/ml)[a]	CD4 (cells/mm^3)	Sample[b]	Residue encoded in place of indicated amino acid															
					65K	67D	69T	70K	75V	101K	103K	108V	151Q	181Y	184M	188Y	190G	215T	219K	225P
BV112	AZT + 3TC + NVP (1 mo)	<50	242	DBS1							K									
				DBS2							K/N									
				DBS3							K									
				BC1							K/N									
				BC2							K									
				BC3							K/N									
				RNA																
BV117	d4T + 3TC + NVP (21 mo)	<50	328	DBS1							K/N									
				DBS2							N									
				DBS3							N									
				BC1							K/N									
				BC2							N									
				BC3							N									
				RNA																

[a] VL, viral load.
[b] DBS1 to DBS3, DBS samples 1 to 3, respectively; BC1 to BC3, blood-cell DNA samples 1 to 3, respectively; RNA, plasma RNA.

The results of the study presented confirmed the feasibility of extracting and subsequently sequencing HIV-1 DNA from DBS as shown by others (24, 43). The sensitivity of our in-house genotyping assay for sequencing DNA from DBS was only slightly lower than the sensitivity for sequencing DNA extracted from whole blood cells. Besides allowing the successful sequencing of the PR and RT gene in 93.1% of the patients with a detectable viral load, the DBS also allowed PR and RT gene sequencing of samples from more than 50% of the patients with an undetectable viral load. The reduced sensitivity compared to that of the patients with a detectable viral load suggests a correlation between the viral DNA contributions in DBS and the plasma viral load, as was reported by McNulty et al. (24). Although one might expect that in patients with a low CD4 count, the total number of HIV-infected cells per volume of blood is limited, no relation was seen between low CD4 counts and failure to amplify viral DNA from DBS or failure to detect resistant strains in the DNA (results not shown). The possibility of obtaining sequence information from patients with an undetectable viral load can be valuable for epidemiological studies and subtype determination and might allow the detection of mutations selected during previous suboptimal treatment regimens, as is discussed below.

If all nucleotides are considered, irrespective of the importance of their position for drug resistance, good correlations between the nucleotide sequences of DBS DNA, whole-blood-cell DNA, and RNA were obtained, with differences that only slightly exceeded the differences observed between replicate sequencing reactions starting from the same DNA or RNA extract. For the detection of mutations at positions associated with drug resistance, however, superiority of RNA sequencing over DNA sequencing was observed. Relying on only the DNA sequence would lead to a misinterpretation of the assumed phenotypic resistance in 6 of the 11 patients with significant resistance in the virus in the plasma, irrespective of whether blood cells or DBS sequences were considered.

The feasibility of using viral DNA for the establishment of drug resistance is still controversial. Some studies reported more mutations in the plasma RNA (6, 10, 21, 24), and others reported more mutations in the cellular DNA (14, 34). Most of these discordances may be attributed to differences in the selection of the samples used for the evaluation. While plasma RNA represents the population of short-lived actively replicating virus, viral DNA from infected cells is composed of a heterogeneous mix of DNA from acutely infected, actively virus-producing cells as well as quiescent cells that form the viral reservoir (13, 27, 40). Due to the limited capacities of current population-based sequencing genotyping methods, minor variants can remain undetectable (38). The virus replicating in plasma in the early stage of resistance development most probably derives from a small number of cells in the reservoir, and therefore, detection of resistant virus in plasma will precede detection in the cells. Since all patients in this study initiated HAART very recently and about half of the 11 patients in whom drug resistance mutations were detected still had a viral load of less than 5,000 copies/ml at the time of sampling, they can be considered early treatment failures. Although the number of patients is limited, a correlation between low viral load and a reduced capacity for the detection of resistance mutations in DNA compared to that of resistance mutations in RNA was observed, further strengthening the hypothesis of a relationship between duration of treatment failure and the ability to detect drug resistance in DNA, as was suggested by Bi et al. (6).

Despite a lack of sensitivity in the early phase of treatment failure, sequencing of viral DNA can be valuable, especially for patients with previous episodes of treatment failure that were not documented by resistance analysis. Interruption of a failing regimen or a failing drug will result in an overgrowth of the drug-resistant variant by the more fit wild type (13, 27, 40). In the cellular reservoir on the other hand, the resistant strains will remain detectable for longer time. Venturi et al. (39) demonstrated the kinetics of resistant and wild-type virus in the RNA and DNA compartment very clearly. They found more mutations in the RNA samples than in the DNA samples in the patients on a failing treatment, while those undergoing treatment interruption showed more mutations in the DNA samples. The same higher level of sensitivity of DNA for the detection of drug resistance mutations in a group of patients undergoing treatment interruption was reported by Devereux et al. (14). Previous exposure to EFV explains the mutation resulting in the presence of 190A that was found in the DNA but not in the RNA of patient BV114 one month after a switch from EFV to LPV. All the other patients that were selected for

this study were assumed to be on their first line of HAART. Nevertheless, programs aimed at evaluating the influence of a short course of NVP or AZT on mother-to-child HIV-1 transmission have run in this region. Previous exposure to NVP therefore seems the most plausible explanation for the observed 103N mutation in the DNA of patient BV112. This, however, cannot explain the resistance pattern for BV117, a male patient. Surprisingly, both patients BV112 and BV117 showed undetectable viral loads while on NVP-based HAART. The possibility of obtaining an undetectable viral load with an NNRTI-based regimen despite the presence of the mutation resulting in K103N has been observed before (11).

The mutation resulting in the presence of 65R seen in samples from patients BV106 and BV132 is typically known as a tenofovir-associated mutation, but its selection by d4T, especially in patients infected with non-B subtypes, has been reported (37). For patient BV132, the 65R variant was seen in one of the DNA samples only. From the sequencing results for the plasma RNA isolated from this patient, it appears that a mixture of wild-type and mutant strains is present, and this may be the cause for the mutation resulting in the presence of 65R to be missed. The other NRTI or NNRTI resistance-associated mutations that were detected in the DNA sequences only (those resulting in the presence of 70R, 75I, 108I, 219E, and 225H) are considered to have only a minor impact on the drug resistance (http://hivdb.stanford.edu).

The finding of PI-associated mutations in the DNA of two patients who were not on a PI regimen was unexpected. Although the occurrence of 33F in the absence of other PI resistance mutations is rare, we assume that its presence in an isolate from patient BV021 is due to a natural polymorphism. The mutations resulting in the presence of 30N and 46I are seen as part of a mixed population in one of the DNA sequences of patient BV011. We cannot exclude the possibility that this was due to sequencing errors.

The discordances seen between the replicate DNA amplification and sequencing reactions indicate that DNA genotyping, especially genotyping performed on DNA preparations containing small amounts of viral DNA, is prone to a founder effect.

In conclusion, the results of this study demonstrate that whole-blood DNA or DBS DNA sequencing can be a very useful tool for epidemiological studies aimed at analyzing the HIV subtype distribution or the overall distribution of drug resistance mutations in a population. For the follow-up of individual patients, on the other hand, replacement of RNA sequencing by DNA sequencing cannot be recommended, due to the high number of missed mutations, especially in the early phase of treatment failure. The use of DNA and DBS sequencing for the detection of drug resistance mutations selected during previous drug exposure deserves further study.

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2.4. Effectiveness of antiretroviral therapy and development of drug resistance in HIV-1 infected patients in Mombasa, Kenya.

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Abstract

Objectives: To evaluate treatment success and drug resistance after short term antiretroviral therapy (ART) guided by clinical parameters and CD4 count only, in the Comprehensive HIV Care Centre (CCC) of Coast Province General Hospital, Mombasa, Kenya.

Methods: 150 patients receiving ART were randomly selected. After determination of the plasma viral load, patients with detectable viral load levels were subjected to genotypic drug resistance testing.

Results: At the time of sampling, 132 of the 150 individuals were on ART for more than 6 months (median 21 months, IQR: 12 – 26). An efficient viral load reduction to below 50 copies/ml was observed in 113 (85.6%) of them. Of the 19 patients with a detectable viral load, sequencing of the protease (PR) and reverse transcriptase (RT) gene was successful in 16. Major PR mutations were absent but mutations associated with drug resistance in RT were detected in 14 of the 16 patients (87.5%). High level resistance against at least 2 components of the ART regimen was observed in 9/14 (64.3%). The 3TC mutation M184V and the NNRTI mutation K103N were most frequent but also the multi-drug resistance Q151M and the broad NRTI cross-resistance K65R were observed.

Conclusion: The results of this study revealed a high success rate of short term ART in patients treated in a CCC in a resource-limited setting. Nevertheless, the observed high risk of accumulation of resistance mutations at treatment failure and the selection of multi-drug resistance mutations in some patients is of great concern.

Keywords: HIV-1, antiretroviral treatment, viral load, resistance, resource-limited settings

Introduction

Recent data show an HIV prevalence in Kenya of slightly higher than 5% [1], resulting in about 1 million Kenyans living with HIV today. The number of individuals on antiretroviral therapy (ART) has increased from 3 000 in 2002 to 54 000 in 2005, representing about 20% of those in need of treatment [2]. Comprehensive HIV care, including counselling, prevention and treatment of opportunistic infections, as well as provision of antiretroviral drugs, is currently available in the national referral hospital, in 8 provincial hospitals and in 70 district hospitals across the country [2]. The country's goal is to reach 75% of people in need of ART by 2010.

At the Comprehensive HIV Care Centre (CCC) in Mombasa, decision on when to start or switch treatment is based on clinical and immunological parameters (CD4 count and total lymphocyte count) [3, 4]. Reports on the efficacy of treatment, guided by clinical parameters and CD4 count, as well as on the occurrence of drug resistance in these settings, are scarce. The aim of this study was to assess, in a cross-sectional survey, the rate of viral suppression and drug resistance in a number of randomly selected individuals on ART.

Materials and Methods

Patients and samples

The CCC at Coast Province General Hospital, Mombasa, Kenya, has been operational since 2003. Currently, about 7 000 HIV seropositive patients are registered, among whom nearly 3 000 are receiving ART. The patients are regularly seen by the CCC staff and receive the standard of care following the Kenyan National Guidelines for antiretroviral drug therapy [3]. ART treated patients attending the CCC between 20 April 2006 and 27 April 2006 and between 8 May 2007 and 18 May 2007 were consecutively asked to participate in this surveillance study. A total of 150 patients were included. After obtaining informed consent, basic demographical

and clinical data was obtained from each patient. Adherence was measured by self report and pill count recorded as satisfactory (>95%) or unsatisfactory (<95%). Ten ml of EDTA blood was collected for CD4 cell count (FACScount Becton & Dickinson Immunocytometry, Oxford, UK). The remainder of the EDTA blood was centrifuged to collect plasma which was stored at -80°C until processing for viral load measurement and sequencing.

Roche Amplicor HIV-1 Monitor 1.5 Assay

Plasma HIV RNA quantification was performed, using the Ultrasensitive Cobas Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Basel, Switzerland) with a lower detection limit of 50 copies/ml.

Amplification and genotyping of PR and RT genes

Extraction, amplification and genotyping of HIV RNA was performed by nested RT-PCR and a home-made sequencing assay as previously described [5]. All validations and subsequent manipulations of the sequencing results, as well as the interpretations of the genotyping data and the subtyping were performed using the SmartgeneTM HIV software packages (Integrated Database Network System, Smartgene, Zug, Switzerland). Selection of drug resistance mutations was based on the recent update of the IAS-USA list [6].

Statistical analysis

All statistical analyses were performed using SPSS 15.0 (SPSS, Illinois, USA).

Results

Baseline characteristics of the patients

The median age of the participants was 37 years (IQR: 32 - 43) with 69.3% being women. The majority (66.7%) of participants were in WHO clinical stage 3 or 4 [7]. Baseline CD4 counts were available for 146 participants with a median of 112 cells/mm³ (IQR: 63 – 184). A combination of d4T+3TC+NVP was the most commonly prescribed first line regimen (n=79), followed by d4T+3TC+EFV (n=63), AZT+3TC+NVP (n=5), AZT+3TC+EFV (n=2), and d4T+ddI+EFV (n=1). Patients receiving their first ART regimen had been treated for a median of 17 months (IQR: 10 – 24). In 16 patients treatment was changed after a median of 18 months (IQR: 13 - 26) by switching one (n=7), two (n=5) or three (n=4) drugs because of adverse events including lipodystrophy and/or peripheral neuropathy (n=8), start of anti-tuberculosis treatment (n=1), unavailability of drugs (n=1), or immunological failure (n=6).

HIV RNA viral load quantification, CD4 count and adherence

Eighteen of the 150 patients initiated ART less than 6 months (median 3.5 months, IQR: 2 – 6) before blood collection. The viral load was detectable in 8/18 with a maximum value of 26 300 copies/ml. These patients were excluded for further analyses.

For the 132 patients on ART for more than 6 months, an undetectable viral load was seen in 110 (87.3%) of the 126 patients without treatment changes or with treatment changes for other reasons than immunological failure; and in 3 of the 6 patients who had a treatment switch because of immunological failure. The median viral load of the 19 patients with ongoing viral replication was 3 060 copies/ml (IQR: 294 – 21 000).

A detectable viral load was not significantly associated with the mean duration of ART (*P*=0.420) or mean baseline CD4 (*P*=0.177).

Results of CD4 counts performed on the samples taken for this study were available for all 132 patients. A significantly higher mean CD4 count was seen in those patients

with an undetectable viral load (n=113, mean=344 cells/mm^3) compared to those with a detectable viral load (n=19, mean=253 cells/mm^3) (*P*=0.032). However, the mean increase in CD4 from baseline level was not significantly different between the two groups (*P*=0.329).

Seventy-four patients (58.6%) reported to have good adherence (>95%). Good adherence was significantly associated with treatment success (*P*=0.020).

Genotypic resistance

Sequencing of the viral RNA was attempted on all 19 samples with a detectable viral load and was successful for PR in 17 (89.5%) and for RT in 16 (84.2%). Amplification failed in 3 patients, all with a very low viral load (79, 81 and 115 copies/ml, after respectively 30, 41 and 10 months of treatment). The subtype distribution for these 17 samples was as follows: subtype A (n=11), subtype C (n=2), CRF16_AD (n=2), subtype D (n=1) and an undefined recombinant in one patient.

No major PR resistance mutations were seen in any of the sequences, but a mean of 4 minor PR mutations were observed per sample (data not shown). Resistance mutations in RT were detected in 14 out of the 16 patients. Overall, the M184V was the most commonly observed mutation (n=12), followed by K103N (n=9). In one patient the multi-drug resistant Q151M mutation was seen and two patients carried virus with a K65R mutation (table 1). In 10 patients a combination of the M184V and at least one mutation conferring NNRTI class resistance was observed.

The two patients who presented with a wild type virus had a viral load of 533 and 1380 copies/ml after 23 and 26 months of treatment respectively.

Discussion

The intensive campaign of WHO to improve the availability of ART worldwide is gradually beginning to pay off and most African countries are currently able to

provide first line ART regimens to a considerable number of HIV-1 infected individuals who need medication. Efforts to scale-up laboratory facilities for monitoring treatment in these patients however are running behind. A random sample survey is often the only way to assess treatment efficacy and the selection of drug resistance in comprehensive care treatment programs in Africa. The information obtained from these surveys is of high importance for the eventual future adaptation of treatment strategies. Moreover, resistance data obtained from these surveys will be crucial in evaluating the value of second line treatment regimens in Africa where still only a limited number of drugs are available today.

We sampled 150 individuals receiving ART and found a relatively high percentage (82.0%) of complete viral suppression (VL<50 copies/ml), with 85.6% of 132 patients treated for more than 6 months having an undetectable viral load. These figures are even higher than the ones obtained with similar regimens in the US and Europe [8-12], but comparable to what has been published for other African regions [13-16]. Only Spacek *et al*. reported a considerable lower rate of viral suppression (66%) in Uganda, but in their study patients had to pay for their medication, which might have negatively influenced drug adherence [17]. Indeed, a meta-analysis has confirmed a higher success rate of virological suppression when free medication is provided [18].

The high treatment success rate seen in this study might be partly due to a bias because of a possible selection of patients who attend the CCC more regularly. These patients could be the more motivated individuals having a better treatment adherence. Although the total number of patients was small, a significant correlation between adherence and treatment success was demonstrated.

Despite the high efficacy of the treatment, an exceptional high percentage of the patients that were found with a detectable viral load after more than 6 months of treatment, presented with drug resistance mutations.

The combinations of AZT or d4T with 3TC and EFV or NVP are extensively used as a first line regimen in resource-limited settings [19]. Both 3TC and the NNRTIs EFV and NVP have a low genetic barrier towards resistance and it is therefore not unexpected that, in accordance with the results of other studies, the 3TC mutation M184V and the NNRTI mutations K103N, G190A and Y181C are frequently observed in case of treatment failure [10, 14]. The high percentage (62.5%) of patients with a combination of M184V mutations and at least one NNRTI resistance associated mutation, as well as the selection of the broad NRTI cross-resistance mutations Q151M and K65R in 3 patients are worrying. Despite the presence of the thymidine analogues AZT or d4T in most of the regimens, thymidine analogue mutations (TAMs) were infrequently observed.

Accumulation of mutations against drugs from different drug classes and/or the presence of broad cross-resistance mutations will jeopardize the effectiveness of recommended second line regimens that often include ABC and TDF. Moreover, the limited availability and the high cost of second line antiretrovirals force clinicians in resource-limited settings to recycle drugs. Based on the resistance data from this study, we can assume that the effect of second line regimens with recycled drugs will be at the most temporary.

The small number of patients on a second line regimen that were included and the limited time period between initiation of this regimen and the date of sampling, did not allow us to make conclusions about the efficiency of second line regimens.

In conclusion, the results of this observational study show that effective first line ART treatment in clinical care centres with limited resources is feasible. However, the resistance data presented point out the danger of the currently recommended first line regimens, when used in the absence of virological monitoring, with regard to the accumulation of resistance mutations. The observed high percentage of genotypic drug resistance against at least two drugs, questions the usefulness of a second line

treatment considering the lack of choice for drugs and the absence of guidance by resistance testing.

Besides high quality adherence counselling, efforts to guarantee a robust supply of several antiretroviral drugs from different classes and the worldwide availability of affordable and simple viral load and genotyping assays are needed to prevent the current success of global ART programs from being only temporary.

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Table 1: Overview of the resistance mutations detected in the RT gene of patients with a detectable viral load after more than 6 months of ART.

ART: antiretroviral therapy, VL: viral load, c/ml: copies/ml

Sample ID	Subtype	ART	Time	VL (c/ml)	CD4 (cells/mm³)	65K	67D	69T	75V	103K	108V	151Q	181Y	184M	188Y	190G	215T	225P
BV123	A1+D	d4T+3TC+NVP	8m	3060	321					N				V				
BV105	C	AZT+3TC+EFV	9m	21000	276					N				V				
BV275	-	d4T+3TC+NVP	10m	115	212	Sequencing not successful												
BV133	A1	d4T+3TC+NVP	10m	>100000	165							M		V	L	A		
BV264	A1	d4T+3TC+NVP	12m	78600	180	R			I				C	V		A		
BV141	A1	d4T+3TC+NVP	13m	10400	388					N				V				
BV147	CRF16_AD	d4T+3TC+EFV	13m	98200	218					N				V			F	H
BV116	A1	d4T+3TC+NVP	22m	>100000	353					N				V				
BV209	A1	d4T+3TC+EFV	23m	533	160													
BV106	A1	d4T+3TC+NVP	23m	3520	300	R								V				
BV255	A1	d4T+3TC+NVP	26m	1380	83													
BV232	D	d4T+3TC+NVP	26m	10100	149					N/K			C/Y	V		A/G		
BV245	A1	AZT+3TC+EFV	27m	83	180					N								
BV224	-	d4T+3TC+NVP	30m	79	515	Sequencing not successful												
BV113	A1	d4T+3TC+EFV	32m	1030	467		N							V				
BV145	A1	d4T+3TC+EFV	33m	294	206					N	I			V				
BV223	A1	d4T+3TC+EFV ABC+3TC+LPV	18m 1m	1370	188									V		S		
BV114	CRF16_AD	d4T+3TC+EFV AZT+3TC+LPV	34m 1m	15300	217					N								H
BV120	-	AZT+3TC+NVP d4T+3TC+LPV	41m 9m	81	226	Sequencing not successful												

Chapter III: Discussion and future perspectives

3.1. Discussion

More than 25 years after the first case of HIV/AIDS and more than 10 years after the first use of HAART, the worldwide AIDS epidemic remains a challenge. At the end of 2007, about 33 million people were living with HIV/AIDS, with nearly 2.5 million newly infected yearly [1]. The majority of them reside in Sub-Saharan Africa and Asia. Despite worldwide efforts to prevent the HIV spread, the epidemic continues to grow. In the last couple of years the delivery of HAART in low and middle-income countries has been a public health priority. The World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) launched the '3 by 5' initiative in December 2003. This programme aimed at closing the gap between those in need of treatment and those receiving it. Substantial progress has been made in expanding the provision of treatment in resource-limited settings, although results were less optimal than initially hoped for: in June 2006 about 2 million people received treatment, which is only 28% of those who need it [75]. This result is sobering and encouraging at the same time; decent health care infrastructure, trained staff and affordable drug supplies are still lacking in many of these countries, nevertheless some resource-limited countries showed that these obstacles can be overcome. The new goal now is to get as close as possible to universal access of treatment by 2010.

In industrialised countries, CD4+ T cell counts, viral load assays and resistance assays are widely recommended and used to monitor HIV-infected patients on treatment. Yet, due to limited resources and inadequate laboratory infrastructure in most resource-limited countries, laboratory monitoring for patients on treatment has been kept to a minimum and priority was given to the improvement of the widespread availability of antiretroviral drugs [77]. CD4+ T cell assays, which are important laboratory tools to initiate treatment [39] become more widely available in resource-limited countries, but the supply of reagents remains challenging in most places. Although CD4 count is considered as a suboptimal marker for ART monitoring, compared to the gold standards of viral load and resistance testing, the current WHO recommendation is to switch to a second-line treatment based on clinical or immunological failure [39]. This has led to significant misclassification of therapeutic responses as was pointed out by some studies [83-87] and a much wider introduction

of viral load measurements for ART monitoring is urgently needed. This can only be implemented if the costs of these assays can be reduced and if technical issues can be overcome in resource-limited settings.

Since delivery of ART in resource-limited countries is expanding, the need for viral load assays is increasing [123]. Regular viral load determination allows the early recognition of treatment failure and, if necessary, a timely therapy switch to avoid accumulation of resistance mutations and development of broad cross-resistance, or an early adherence intervention in case of viral load rebounds [45, 46, 124]. This is not only important for the individual patient, the spread of resistant viruses in the population can also jeopardise future treatment for recently infected, ART naïve patients. Besides using viral load measurements for treatment monitoring, it can also be used for the diagnosis of perinatal infections by detection of viral RNA [88-90, 125] and as a sentinel surveillance tool in ART program quality assessments. To achieve these goals, the costs of these assays have to decrease substantially.

Besides the problem of the high cost of reagents and equipment, the implementation of viral load testing in resource-limited settings is often hindered by the lack of decent laboratory infrastructure (clean water, electricity, air conditioning etc.), trained lab technicians, sample shipment facilities, equipment maintenance service and external quality control programs. Although it will be extremely difficult to find an assay that is at the same time sensitive, specific, easy to perform and cheap, some alternative assays have been developed and evaluated. Schupbach and colleagues were the first to suggest an alternative viral load assay by quantitatively measuring p24 Ag [91, 96, 97, 126]. The ultrasensitive p24 Ag assay (Perkin-Elmer) measures the p24 Ag in plasma by a standard ELISA test. The promising results of this simple and cheap assay (5-10 USD/test) were confirmed by some [92-95, 98], but others showed a lack of correlation between the p24 assay and the standard viral load assays [99-101]. Due to conflicting results and the need for an external lysis buffer to increase sensitivity, the p24 Ag assay is no longer recommended to monitor patients on treatment. However, this assay is suitable for paediatric diagnosis of HIV-infection [102-105]. Another non-nucleic acid-based alternative for measuring HIV-1 viral load is the ExavirLoad assay (Cavidi), that measures HIV RT enzyme activity by an ELISA assay [108]. Correlations between ExavirLoad and FDA approved viral load assays seem to be more consistent, including high sensitivity and specificity [98, 109-113, 127, 128]. A third group of alternative viral load assays are the in-house real-time

PCR assays. Lewin *et al.* were the first to describe such an assay to detect HIV-1 RNA [114] and others showed that the quantification of HIV-1 RNA by real-time PCR was feasible [115-120]. Rouet *et al*, found their in-house real-time PCR assay to be suitable for patient monitoring and paediatric diagnosis in Côte d'Ivoire [119]. Based on these good results, a French company (Biocentric) decided to produce 'Generic HIV viral load assay' to quantify HIV-1 RNA.

At the moment, the ExavirLoad and a real-time PCR assay, like the Generic HIV viral load, seem to be the most promising alternatives for the standard viral load tests. Few groups have evaluated these assays in resource-limited settings and most of the evaluations were done in collaboration with the manufacturer. The availability of both assays in Coast Province General Hospital in Mombasa made us decide to assess them in a real-life situation and to compare the results of both assays with the results of the standard viral load assay from Roche. We found a good correlation for both assays when compared with the Amplicor HIV-1 Monitor v1.5 assay, but the specificity was less optimal, especially for the ExavirLoad. As the quantification of HIV-1 RNA by ExavirLoad is based on RT enzyme activity little influence of different subtypes is expected [108, 110]. However, in our study, a relatively higher number of false positive results were seen for subtype C and D. Further analysis was limited due to the small sample size and after pooling the non-A subtypes no statistical difference between non-A and subtype A could be detected. The Generic HIV-1 viral load deals with the HIV-1 diversity challenge by targeting the non-specific LTR region [117, 118]. No subtype-related differences in results were detected in our study, confirming the suitability of the LTR region for broad subtype specific amplification assays.

Both the Generic HIV viral load assay and the ExaVirLoad assay could be valuable tools for viral load determination in resource-limited settings, yet both tests have their limitations and should not be implemented without a thorough on-site evaluation. The ExavirLoad has the advantage of a low start-up cost (3000 USD) and virtually no maintenance requirements, but despite the relatively simple method, the assay is time consuming, the cost per test remains relatively high (30 USD/test) and no external quality control program has been developed yet. The Generic HIV viral load on the other hand, has a low turn-around time, a relatively low cost per test (10-20 USD) and an established external quality control program (Agence Nationale de Recherches sur le SIDA, ANRS Paris), but the assay requires expensive equipment

that needs regular servicing. This indicates that the choice for the right viral load assay should be made at each site individually, taking the available facilities and specific needs into account. Meanwhile manufacturers of commercial viral load assays should commit themselves to further simplify their assays, reduce the prices and provide the necessary support in infrastructure for the use of their assays in resource-limited settings.

Besides viral load assays, current US and European treatment guidelines recommend genotypic resistance assays to assess the presence of baseline resistance before treatment initiation, and in all cases where treatment failure is suspected [36-38]. The information obtained by genotypic resistance assays can assist in the selection of the optimal drug regimen. However the high cost and complexity of these assays make the tests almost inaccessible in resource-limited settings. Moreover, one might argue that the need for drug resistance assays in resource-limited settings is not extremely urgent yet, because the expected resistance pattern is predictable, when failing the widely used NNRTI-based first-line regimens [129, 130] and guidelines recommend to change all components of the regimen in case of failure [39]. In reality however, access to second-line drugs remains a problem in resource-limited settings. Due to the few ARV drugs available in these regions, replacing all the components of the first-line regimen is often impossible and recycling drugs in the second regimen remains the only option. In this case, the efficiency of the second-line regimen will definitely improve if the choice of the medication can be guided by resistance testing. The need for drug resistance assays will increase even more when the number of available ARV drugs in resource-limited settings increases and when third-line and salvage regimens become an option. Because viral load assays and potent second-line regimens are not always available, many patients in resource-limited settings will be exposed to sub-optimal treatment, increasing the risk of accumulation of resistance mutations and the probability of cross-resistance. This will not only pose a problem for the individual patient, but also increase the risk of transmission of highly resistant viruses in the population. The prevalence of transmitted drug resistance (TDR) in countries with established ART programs ranges from 6.0% to 24.1% in the US and Western Europe and from 3.1% to 22.2% in Southern America [47]. At the moment the prevalence of TDR in resource-limited settings is still low [47], but some fear that the

numbers are likely to increase now that ART is more widespread and virological failure might only be detected at a later stage. However, due to the absence of mono- and bitherpay, the rate of transmitted drug resistance will be lower than that seen in the industrialized world. Blower et al. have predicted that the transmission rate of drug resistant HIV in resource-limited settings will be below the WHO surveillance threshold of 5%, assuming that 10% of the HIV infected population is treated [131]. On the other hand, a high proportion of treated cases can be expected to develop drug resistance, and therefore it is suggested that sentinel surveillance to monitor TDR should mainly be done in a few areas where treatment coverage is high. Monitoring the prevalence of drug resistance in treated individuals would be an effective strategy to monitor program effectiveness and to asses the public health impact of the roll-out of ART on drug resistant HIV [131].

This shows that there is a need for affordable genotypic resistance assays and we therefore assessed an in-house assay that allows the combined detection of plasma HIV-1 and drug resistance. In this assay the protease (PR) and reverse transcriptase (RT) gene, two major target genes for antiretroviral therapy, were amplified in a highly sensitive nested RT-PCR and subsequently used in a direct sequencing procedure. The high sensitivity (100% for samples with a viral load >500 c/ml and about 75% for samples with a viral load between 50 and 500 c/ml) indicates that this subtype-independent assay can potentially be used as a qualitative viral load assay (25 USD/test), reducing the need for a standard viral load assay. Positive PCR results, indicating treatment failure, can subsequently be used to detect possible drug resistance mutations (40 USD/test). The use of this method for the follow-up of patients under treatment will allow fast detection of emerging drug resistance at a more affordable price than the commercial tests.

Although the cost of drug resistance assays can be reduced by using in-house methods, the need for specialised equipment and proper infrastructure remains. This is not only a problem for the assay itself, but also the collection of samples is subject to logistical obstacles like storage and transportation in a cold chain. If the need for a cold chain can be avoided, samples could easily be collected in the field and then sent to a centralised reference laboratory where the required equipment and infrastructure are available. Dry blood spots (DBS) are an ideal medium for blood collection in the field. A drop of blood can be spotted directly on the filter paper and

after air drying the papers can be handled as non-infectious and transported to a central laboratory by regular air mail [132]. Moreover, this method of sample collection avoids the need for centrifugation, the need for a freezer at the collection site and the need for frozen shipment. In the last 25 years DBS have been used for various purposes, including detection of HIV antibodies [133-137], detection of HIV antigen [138, 139], early diagnosis of perinatally infected infants [140-144], quantification of CD4+ T cells [145] and quantification of viral plasma RNA [146-153]. Few studies examined the possibility to perform drug resistance testing on dried blood, plasma or serum on filter paper[154-156]. Our in-house sequencing method for RNA was adapted for the use of DNA from whole blood and DBS. The high sensitivity that was found, confirmed the feasibility of extracting and sequencing HIV-1 DNA from DBS as previously shown by others [156, 157]. Because DNA is a more stable molecule compared to RNA and DNA requires less manipulations, its use is ideal in resource-limited settings. However, the correlation between drug resistance-related mutations detected in RNA and DNA remains unclear. Despite the fact that most studies found a substantial correlation between DNA and RNA, some studies detected more mutations in DNA [158, 159] while others reported more resistance mutations in RNA sequences [157, 160-163]. Also in our study, we found more mutations in RNA sequences compared to DNA, especially in patients with a relatively low viral load. This can be explained by the different kinetics between the plasma RNA and the whole blood DNA compartments as the genetic turn-over of PBMCs (DNA) is much slower compared to plasma RNA and only a minor subset of PBMCs is involved in the HIV replication [16, 17]. As a result, the emergence of resistance related mutations in plasma RNA precedes that in DNA and sequencing of RNA remains superior in the detection of resistance at early treatment failure. Despite the poor correlation between RNA and DNA in our study, sequencing from DNA can still be a valuable tool for the detection of mutations selected under previous drug regimens [158, 159] and in a more epidemiological approach to analyse the HIV subtype distribution and the spread of drug resistance mutations in a population.

Despite the lack of accurate laboratory tools to monitor ART treated patients in resource-limited settings, the scale-up of ART has been proven successful in these regions [129, 164-168]. In our study in Mombasa, the treatment success rate was

86.2% for those treated for at least 6 months. Some of the reasons for these successful early outcomes are the availability of highly effective HAART for treatment naïve patients [76], the low prevalence of primary resistance [47] and the unexpectedly high level of treatment compliance [169]. In most resource-limited settings, the decision on when to start or switch treatment is solely based on clinical and immunological markers [39]. For this reason, patients are likely to remain on a failing regimen for a longer period, thereby increasing the risk of accumulating drug resistance mutations in the virus, which jeopardizes second-line treatment options and which increases the risk of spreading transmitted drug resistance in the population. The accumulation of resistance mutations under these circumstances has been proven by several groups [129, 166, 170] and was confirmed by our study where 87.5% of the patients with a detectable viral load developed drug resistance mutations in the RT gene. Due to the low genetic barrier, the development of M184V and K103N mutations is predictable under the recommended first-line regimens of AZT or d4T with 3TC and NVP or EFV [129, 130]. Nevertheless, it is of great concern that more than 60% of the patients with virological failure after a relatively short time developed high level resistance against 3TC and the NNRTI class. Moreover, in three patients the multi-drug resistant Q151M and K65R mutations were detected. This observation will have major implications towards the selection of a potent second-line regimen. Ideally, a second-line regimen should consist of at least three fully active drugs in order to obtain complete viral suppression [36-38]. In the absence of drug resistance assays in resource-limited settings, the best option would be to start a second regimen with 3 not previously used drugs, having low cross-resistance with the drugs that were taken before. At the moment this does not seem to be a very feasible option in many resource-limited settings, due to the limited availability of second-line drugs. A better accessibility to second-line regimens should be a top priority. A Thai study demonstrated that up to 48% of the patients failing their first-line regimen have limited options for second-line treatment with the currently available drugs in their country [170]. Therefore it is likely that a number of drugs will be recycled. Especially the NRTI 3TC is considered to be a possible candidate for recycling. Although 3TC has a significantly reduced activity in patients harbouring virus with the M184V mutation, some evidence exists that viruses harbouring this mutation partly lose their replicative capacity [171] and that partial antiviral activity of 3TC is preserved [172, 173]. Moreover, the development of the

M184V mutation can resensitize the virus for AZT in case of the presence of the AZT resistance mutation 215Y [174]. At this stage, the long term benefits of the M184V mutation are less well understood. Nevertheless, when no options for a fully suppressive regimen are available, a 3TC-based regimen can be continued. Sungkanuparph et al. also indicated that the number of patients failing their first-line regimen with limited second-line options increased to 63% among patients with late detection of treatment failure and decreased to 30% when treatment failure could be detected early [170]. In our study three patients still carried a wild type virus at treatment failure (viral load: 55, 533 and 1380 c/ml), after being treated for respectively 6, 23 and 26 months. Again this advocates for the implementation of viral load assays in order to detect early treatment failure and prevent the development of extensive drug resistance as much as possible. Although this approach will increase the laboratory costs, it will reserve scarce and expensive second-line regimens for those who really need it.

There is still a long way to go in order to improve the treatment and care of HIV infected patients. Continuous efforts should be made to further expand the access to ART in middle and low-income countries and thereby special attention should be give to the monitoring of these patients on treatment.

While affordable ART becomes gradually more and more available in many resource-limited settings and the necessity for affordable and field-adapted monitoring tools seems to get on the local and international agendas, it is of uttermost importance to continue the search for new and the implementation of existing preventive strategies.

After more than 25 years in the HIV epidemic, it seems that the main preventive tool remains the male condom. Given its correct use, this device provides very good protection against HIV and other sexually transmitted infections. Unfortunately, it is not easy to motivate people to use condoms, and on top of that, the condom is a male controlled prevention tool leaving the women in a vulnerable position. Therefore there is an urgent need for better preventive strategies. A major success in the field of prevention was recently obtained when male circumcision proved to reduce the number of new infections by at least 50% in circumcised men [175-177].

An HIV vaccine would provide the best solution to prevent the epidemic to spread even more, but the chances to find a safe vaccine with good efficacy within the next 10 years seems to be further away than ever, after the recent closure of the clinical

trials for the Merck's STEP adenovirus-5-based vaccine [178, 179].The development of an effective microbicide is another important track to prevent further spread of HIV. This prevention method is female-controlled which is an important characteristic. Unfortunately, after nonoxynol-9 was found to be ineffective to prevent HIV infection, and even possibly harmful when used as a microbicide [180], another possible candidate joined the list of failures. Last January, a phase III clinical trial with cellulose sulphate was closed early because preliminary results indicated this product could potentially lead to an increased risk of HIV acquisition in those women using the product. In response another trial using cellulose sulphate in Nigeria was stopped as a precaution [181]. Despite this negative signal, there is still hope as several other microbicide candidates, using different modes of action, are currently in phase II and III clinical trials [182].

3.2. Future perspectives

The initial doubts about the implementation of ART roll-out in resource-limited settings have been proven wrong. Many countries have shown that the logistical challenges of public health infrastructure, training of staff and the supply of antiretroviral drugs can be overcome, but there still lies a long way ahead of us. The need for affordable viral load and genotyping assays is increasing in order to further improve the quality of care provided to the majority of HIV-infected individuals. Viral load assays are not only useful for detection of early treatment failure, but they can also be of great value in monitoring treatment adherence, in the diagnosis of perinatally infected children and in general efficacy assessments of ART roll-out programs. The simple and cheaper alternative viral load assays that are currently on the market still have their specific limitations including sensitivity/specificity, high start-up costs, need for specialised equipment and trained personnel. Moreover, their use in the follow-up of patients on treatment should be further evaluated. Furthermore, the continuous search for less complicated and low-cost alternatives based on dipstick technology [183], micro-arrays [184] or molecular zipper [185] are of extreme importance. Ideally, new viral load assays should be so simple that they can be used in district health care settings. Meanwhile, companies marketing viral load assays should make an effort to reduce prices for their tests in resource-limited settings.

Even if viral load assays would become more readily available and early treatment failure can easily be detected, the development of drug resistance is inevitable. Therefore the need for genotypic resistance assays is also important. Options for simultaneous detection of viral load and possible resistance mutations, like our in-house method, should be further explored. The implementation of our in-house genotyping assay should be evaluated in a resource-limited setting such as Mombasa because the combined detection of possible virological failure and subsequent identification of potential drug resistance mutations is a promising strategy. In this respect we could set-up a study whereby patients on ART are monitored using two systems: first, the standard follow-up system (standard viral load assay and subsequent genotyping in case of a detectable viral load) and secondly, our in-house genotyping assay whereby the qualitative amplification step replaces the standard viral load assay. When this in-house genotyping system proves to be

equally useful in the monitoring of patients on treatment, the implementation of this assay in a resource-limited setting, such as Mombasa, should be evaluated.
The optimisation of genotypic resistance assays on RNA and DNA extracted from dry blood spots deserves more attention as its use would simplify sample collection on site and sample shipment to central laboratories.

But let us not make the perfect the enemy of the good. Despite the limited availability of adequate laboratory tools to monitor patients on treatment and the relatively high rate of resistance among patients failing treatment, the roll-out of ART in resource-limited settings should be continued in order to close the gap between the many in need of treatment and the few who actually have access to it. Continuous monitoring of ART programs in terms of treatment success and development of resistance should be used for future adaptations of treatment strategies and more ARV drugs should be generically made available in resource-limited areas, including newly developed drugs.

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Curriculum Vitae

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Education

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1993-1999 Secondary school, Sciences-Mathematics
Humaniora Virga Jesse, Hasselt, Belgium

Courses

2005 Principles of STD/HIV Research
University of Washington Center for AIDS and STD
Seattle, Washington, USA (18-28 July)

ABI PRISM 7000 SDS course (Applied Biosystems)
Mombasa, Kenya (8-9 February)

2004 Introduction to SPSS
Institute for permanent Education in Sciences (IPVW)
Ghent University, Belgium (4-8 October)

Workshop on Good Laboratory Practices (GLP)
Mombasa Medical College, Mombasa, Kenya (10 June)

2003 Course for intercultural training and international cooperation
ITECO (July)

Publications

J. Snoeck, C. Riva, **K. Steegen**, Y. Schrooten, B. Maes, L. Vergne, K. Van Laethem, M. Peeters and A.-M. Vandamme, 2005. **Optimization of a genotypic assay applicable to all human immunodeficiency virus type 1 protease and reverse transcriptase subtypes.** *J Virol Meth* 128: 47-53.

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K. Steegen, **S. Luchters, E. Demecheleer, K. Dauwe, K. Mandaliya, W. Jaoko, J. Plum, M. Temmerman and C. Verhofstede, 2007** Feasibility of detecting HIV-1 drug resistance in DNA extracted from whole blood or dry blood spots. *J Clin Microbiol* 45 (10): 3342-3351

Conferences attended

13th International Conference on AIDS and STIs in Africa, Nairobi, Kenya, 21-26 September, 2003

5th European HIV drug resistance workshop, Cascais, Portugal, 28-30 March 2007

1st INTEREST Workshop: International workshop on Interventions in HIV Treatment, Pathogenesis and Prevention Research in Resource Poor Settings, Kampala, Uganda, 30 May-2 June 2007

International ICRH Conference: Sexual and Reproductive Health Research: Making a Difference, Ghent, Belgium, 19 October 2007

Oral presentations

Laboratory diagnostics and immunological tests: rapid testing, CD4/CD8 and viral load
National AIDS and STI Control Program (NASCOP) Kenya: ARV management training for health care provider. Plaza Beach Hotel, Mombasa, 5th-9th April 2004

Evaluation of the Usefulness of DNA from whole blood or dried blood spots for the detection of drug resistance mutations (abstract 55), *1st INTEREST workshop, Kampala, Uganda, 30 May-2 June 2007*

Laboratory tools for treatment management of HIV-1 infected patients in resource-limited settings*, International ICRH Conference: Sexual and Reproductive Health Research: Making a Difference, Ghent, Belgium, 19 October 2007*

Posters

K. Steegen, S. Luchters, N. De Cabooter, J. Reynaerts, K. Mandaliya, W. Jaoko, J. Plum, M. Temmerman and C. Verhofstede. **High level of HIV drug resistance in HAART treated patients attending the HIV Comprehensive Care Centre in Coast Province General Hospital, Mombasa, Kenya.** (poster 7)**,** *5th European HIV drug resistance workshop, Cascais, Portugal, 28-30 March 2007*

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